The University Of Sheffield.

Access To Thesis.

- A bound copy of every thesis which is accepted as worthy for a higher degree, must be deposited in the University of Sheffield Library, where it will be made available for borrowing or consultation in accordance with University Regulations.
- All students registering from 2008–09 onwards are also required to submit an electronic copy of their final, approved thesis. Students who registered prior to 2008–09 may also submit electronically, but this is not required.

Author: Laleh Dehghan Marvast Dept: Human Metabolism

Thesis Title: The role of C-terminal Agrin in intracellular complex. Registration No: 07023791

For completion by all students:

Submit in print form only (for deposit in the University Library): ☑

Submit in print form and also upload to the *White Rose eTheses Online* server: In full ☑

Edited eThesis ☑

Please indicate if there are any embargo restrictions on this thesis. Please note that if no boxes are ticked, you will have consented to your thesis being made available without any restrictions.

Embargo details: (complete only if requesting an embargo to either your print and/or eThesis)	Embargo required?		Length of embargo (in years)
Print Thesis	Yes ☑	No ☐	1 year
eThesis	Yes ☑	No ☐	1 year

Supervisor: I, the supervisor, agree to the named thesis being made available under the conditions specified above.

Name: ALLAN PACEY Dept: HUMAN METABOLISM

Signed: [signature] Date: 20th JUNE 2013

Student: I, the author, agree to the named thesis being made available under the conditions specified above.

I give permission to the University of Sheffield to reproduce the print thesis in whole or in part in order to supply single copies for the purpose of research or private study for a non-commercial purpose.

I confirm that this thesis is my own work, and where materials owned by a third party have been used copyright clearance has been obtained. I am aware of the University's *Guidance on the Use of Unfair Means* (www.sheffield.ac.uk/lets/design/unfair)

I confirm that all copies of the thesis submitted to the University (including electronic copies on CD/DVD) are identical in content.

Name: Laleh Dehghan Marvast Dept: Human Metabolism

Signed: Laleh Marvast Date: 20th, June, 2013

For completion by students also submitting an electronic thesis (eThesis):

I, the author, agree that the University of Sheffield's eThesis repository (currently WREO) will make my eThesis available over the internet via an entirely non-exclusive agreement and that, without changing content, WREO may convert my thesis to any medium or format for the purpose of future preservation and accessibility.

I, the author, agree that the metadata relating to the eThesis will normally appear on both the University's eThesis server and the British Library's EThOS service, even if the thesis is subject to an embargo. I agree that a copy of the eThesis may be supplied to the British Library.

I confirm that the upload is identical to the final, examined and awarded version of the thesis as submitted in print to the University for deposit in the Library (unless edited as indicated above).

Name: Laleh Dehghan Marvast Dept: Human Metabolism

Signed: Laleh Marvast Date: 20th, June, 2013

THIS SHEET MUST BE BOUND IN THE FRONT OF THE PRINTED THESIS BEFORE IT IS SUBMITTED

The Role of *Chlamydia trachomatis* and *Mycoplasma genitalium* in infertile couples

Laleh Dehghan Marvast

A thesis submitted for the degree of Doctor of Philosophy

Department of Human Metabolism

June 2013

Abstract

Chlamydia trachomatis is one of the most prevalent sexually transmitted diseases. Previous work has shown that *C. trachomatis* IgG antibody is correlated with tubal factor infertility and reduced pregnancy rates. There has been little research on *Mycoplasma genitalium* and the incidence in infertile couples remains unknown. This study has investigated 250 infertile couples as well as 250 fertile women. The presence of these organisms was detected by polymerase chain reaction and an analysis of serum IgA, IgM and IgG antibodies to *C. trachomatis*.

Low prevalence of *C. trachomatis*, low level of concordance within couples and no evidence of *M. genitalium* was found. Elevation of IL6 and 8 were observed in *C. trachomatis* positive men and this varied with diagnostic method. Seminal IL-8 was correlated negatively with semen volume and positively with age. Sperm DNA fragmentation (TUNEL) and chromatin structure (AB, AO & CMA3) were not correlated with *C. trachomatis* infection. TUNEL results correlated negatively with semen volume and the percentage of protamination was related to age. Urethritis was more common in men diagnosed with *C. trachomatis* by urine DNA. Men who work as drivers were more likely to be positive for IgG. PCOS was more common in women with positive serology for IgM and IgG. *C. trachomatis* infection in men or women did not affect pregnancy rate and pregnancy outcome in either assisted or spontaneous conception.

In conclusion, this thesis does not support the hypothesis that there is a strong relationship between *C. trachomatis* infection and infertility. Nor does it support the idea that *C. trachomatis* is linked to poor semen quality. Study limitations include the lack of fertile men as control group and difficulties with some methodology. A number of future studies are suggested along with some recommendations of *C. trachomatis* screening programmes both in Iran and the world.

Acknowledgements

I would like to express my very sincere gratitude to my supervisor Dr Allan Pacey for the systematic guidance and great effort he put into training me in the scientific field with his continuous advice and encouragement throughout the course of this thesis with his excellent patience.

I also acknowledge my gratitude to Dr Adrian Eley who I have been indebted in the preparation of this thesis; he has supported me with his patience, guidance and knowledge, as well as his academic experience.

I would like to thank all those who helped me during my PhD degree, specially the member of Research and Clinical Centre for Infertility, Yazd, Iran. My great thank goes to Prof. Aflatoonian for his support and guidance during patient recruitment. Thanks must also go to Dr Talebi for knowledgeable guidance and Dr Abdoli for his excellent support.

Other people I should acknowledge include Ian Geary (Academic Unit of Infection and Immunity, The University of Sheffield) and Jalal Ghasemzadeh (Andrology Laboratory, Research and Clinical Centre for Infertility, Yazd). Also MASH group at The University of Sheffield for excellent statistical advices.

Last, but not least, my very special thanks go to my family for their support and patience in particular my brother for his support and sponsoring my postgraduate study.

Table of Contents

List of Figures

List of Tables

Abbreviations:

ART	Assisted reproductive treatment
ASRM	American Society for Reproductive Medicine
Ab	Antibody
AB	Aniline Blue
AO	Acridine Orange
BV	Bacterial vaginosis
BFS	British Fertility Society
CASMA	Computer-aided sperm morphology analysis
CD_4	Cluster of differentiation 4
CD_8	Cluster of differentiation 8
CFT	Complement fixation test
CMA3	Chromomycin A3
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CP	Chronic prostatitis
CPPS	Chronic pelvic pain syndrome
CRP	C-reactive protein
DIF	Direct immuno fluorescence
DNA	Deoxyribonucleic acid
EB	Elementary Body
EDTA	Ethylene-diaminetetraacetic acid
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimal Essential Medium
EP	Ectopic pregnancy
ESHRE	European Society of Human Reproduction and Embryology
FCS	Fetal calf serum

FVU	First void urine
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IVF	*In vitro* fertilisation
ICSI	Intra Cytoplasmic Sperm Injection
IUI	Intra Uterine Insemination
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILs	Interleukins
IL-6	Interleukin 6
IL-8	Interleukin 8
IFN	Interferon
ISH	*in situ* hybridisation
LAMP	Lipid associated membrane protein
LCR	Ligase chain reaction
LGV	Lymphogranuloma venereum
MAGI	Male accessory gland infection
MAR	Mixed antiglobulin reaction
MDM	Minimum detectable dose
MgPa	Mycoplasma genitalium protein adhesin
MIF	Micro immunofluorescence
MOMP	Major outer membrane protein
MoPn	Mouse pneumonitis
NAAT	Nucleic acid amplification test
NCNGU	Non-chlamydial non-gonococcal urethritis
NEQAS	National External Quality Assessment Service
NF- κB	Nuclear factor kappa B
NGU	Non-gonococcal urethritis
NHS	National Health Service

NICE	National Institute for Health and Clinical Excellence
NK	Natural killer cell
NPPCR	Nested plasmid polymerase chain reaction
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer solution
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PGU	Post gonococcal urethritis
PID	Pelvic inflammatory disease
PMN	Polymorphonuclear
PPROM	Premature preterm rapture of membrane
QALY	Quality-adjusted life-years
ROS	Reactive oxygen species
RT	Room temperature
SCVS	Self-collected vaginal swab
SDA	Strand-displacement amplification
STD	Sexually transmitted disease
SPSS	Statistical package for the social sciences
TAE	Tris base, acetic acid, EDTA
TB	Tuberculosis
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotidyl transferase
TFI	Tubal factor infertility
TH_1	T helper 1
TH_2	T helper 2
TNFα	Tumor necrosis factor α
TLRs	Toll-like receptors
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TMA	Transcription-mediated amplification
WBC	White blood cells
WHO	World Health Organisation

Chapter 1: General Introduction

1.1 Sexual transmitted disease (STD)

Sexually transmitted diseases (STDs) are considered worldwide as major public health issue, often affecting young adults (Low *et al*., 2006). They can affect male and female fertility, compromise reproductive fecundity and their complications constitute a great socio-economic burden (Piot *et al*., 1988; Cates, 1996). Although there have been advances in managing these infections (Low *et al*., 2006), with regards to the morbidity and mortality associated with them, only limited success has been achieved (Molyneux, 2004). STDs are primarily caused by bacteria and viruses: *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplasma genitalium*, *Treponema pallidum*, are considered bacterial causes of STDs, whereas Cytomegalovirus (CMV), human papillomavirus (HPV), herpes simplex virus type 2 (HSV-2) and human immunodeficiency viruses (HIV) are viral causes. *C. trachomatis, N. gonorrhoeae,* HSV-2 and HIV are the most common STDs with the main health and socio-economic impact (Beagley *et al*., 2000). In this thesis *C. trachomatis* (the most prevalent of the STDs) and *M. genitalium* (a bacterium about which little has been published) have been selected to study.

According to the World Health Organization (2001), 92 million new cases of *C. trachomatis* infection occur every year worldwide with 16 million of new cases found in Sub-Saharan Africa. *C. trachomatis* is the cause of the most prevalent bacterial STD worldwide, and is also recognized as being responsible for at least 50% of an identifiable cause of pelvic inflammatory disease (Scholes *et al*., 1996; Khalaf, 2003). In 87% of untreated men and women there is established persistence of genital chlamydial infections (Joyner *et al*., 2002). In contrast, *M. genitalium* is a STD human pathogen that has been relatively poorly studied. It can lead to urogenital tract disease and has been isolated from the male genital tract in patients with urethritis (Tully *et al*., 1981). *M. genitalium* can act in similar way to *C. trachomatis* in men with non-gonococcal urethritis and women with cervicitis (Gdoura *et al*., 2007).

One of the major causes of female infertility is tubal factor infertility (TFI) and one of the major causes of TFI is damage of Fallopian tubes following infection due to STDs (Clausen *et al*., 2001). Major complications of chlamydial and mycoplasma infections are pelvic inflammatory disease (PID), ectopic pregnancy, infertility, and infant pneumonia. The association between *C. trachomatis* and *M. genitalium* infections and infertility in developed countries has been demonstrated by different studies (Paavonen

& Wolner- Hanssen, 1989; Vigil *et al*., 2002). However, the role of these infections and infertility among infertile couples remains to be determined which this is the focus of this thesis.

1.2 History of *Chlamydia trachomatis*

Chlamydia was discovered in 1907 by Halberstaedter and von Prowazek (Everett *et al*., 1999). They observed it in the epithelium of a conjunctival scraping from an infected orangutan which was inoculated with human trachomatous material (Budai, 2007). Before this discovery, trachoma was well known for thousands of years as a blinding ocular human disease. The Ebers Papyrus from 1550 BC is the first written medical document that mentioned it (Horn *et al.*, 2004). Halberstaedter and von Prowazek (1909) detected an agent similar to trachoma in a case of neonatal blennorrhoea. Heyman and also Frietsch *et al.*, (1910) found trachoma-like inclusions in scrapings from neonate conjunctival and cervical of their mothers. Linder *et al.*, (1911) described the same intracytoplasmic inclusions in infants with ophthalmia neonatorum that was observed in urethral cells of their parents and since then chlamydia was diagnosed as cause of sexually transmitted infections. A description of lymphogranuloma venereum (LGV) pathology and its role in sexual transmission was published in 1913 (Durand *et al*., 1913).

Macchiavello reported the successful culture of trachoma agent in the yolk of an embryonated egg (Macchiavello, 1944). However, trachoma agent culture was confirmed and is usually credited to T'ang and co-workers 1946 (reviewed in Wang, 1999). In 1965 Gordon and Quan reported the use of irradiated McCoy cells for the isolation of *C. trachomatis* and this was a major step in the understanding of chlamydial infections (Ripa, 1982). In comparison with the isolation from eggs, their technique made it possible to perform cultures from genital specimens. The understanding of the basic biology of chlamydia, as well as the clinical aspect of chlamydia, increased following the use of McCoy cells. The role of *C. trachomatis* in infant pneumonia and its involvement in pelvic inflammatory disease (PID) was proposed by Schachter *et al* (1974) and Mardh *et al* (1977). *C. trachomatis* was recognised as an important aetiological factor in acute salpingitis and pelvic inflammatory disease in females (Treharne *et al*., 1979) and after that more studies reported the complications of this organism in women rather than men.

1.2.1 Taxonomy and classification of *C. trachomatis*

Prior to 1999, there was only the genus *Chlamydia* (Moulder *et al*., 1984). Today, the taxonomy currently includes two major genera, *Chlamydophila* and *Chlamydia*, which belong to the family *Chlamydiaceae,* and was recognized in 1999 on the basis of sequence data in the ribosomal genes (Everett *et al*., 1999). The species within the family *Chlamydiaceae* have 16S rRNA gene sequences which are more than 90% identical (Everett and Andersen, 1997; Figure 1.1). The genus *Chlamydophila* includes *Chlamydophila pneumoniae, Chlamydophila pecorum, Chlamydophila psittaci, Chlamydophila abortus, Chlamydophila felis,* and *Chlamydophila caviae*. Species in the genus *Chlamydia* include *Chlamydia muridarum, Chlamydia trachomatis, and Chlamydia suis* (Everett *et al*., 1999).

1.2.2 Developmental cycle

*Chlamydia*e are obligate intracellular bacteria with a unique biphasic developmental cycle. They are unable to synthesis their own ATP (Paavonen and Eggert-Kruse, 1999), thus they need to use their host cell's energy resources to multiply and demonstrate a two- stage developmental cycle of replication (Black, 1997; Paavonen and Eggert-Kruse, 1999). Accumulation of glycogen in inclusions and sensitivity to sulfadiazine are characteristics of *C. trachomatis* (Everett *et al*., 1999). The elementary bodies (EBs) have a complex of disulphide cross-linked envelope proteins which include two cysteine-rich lipoproteins (Everett *et al*., 1999): with a molecular weight 60Kda and second one a low molecular weight (12 Kda). These are called outer membrane complex protein A and B respectively (OmcA and OmcB) and a major outer membrane protein (MOMP) with a molecular weight of 40 Kda (Hatch, 1996; Everett *et al*., 1999). That complex includes Heat–Shock protein in addition (Hatch, 1996; Everett *et al*., 1999). During transformation of elementary bodies (EBs) into reticulate body (RB) chemical reduction in the disulphide cross-links may occur at the time of chlamydial cell infection (Everett *et al*., 1999).

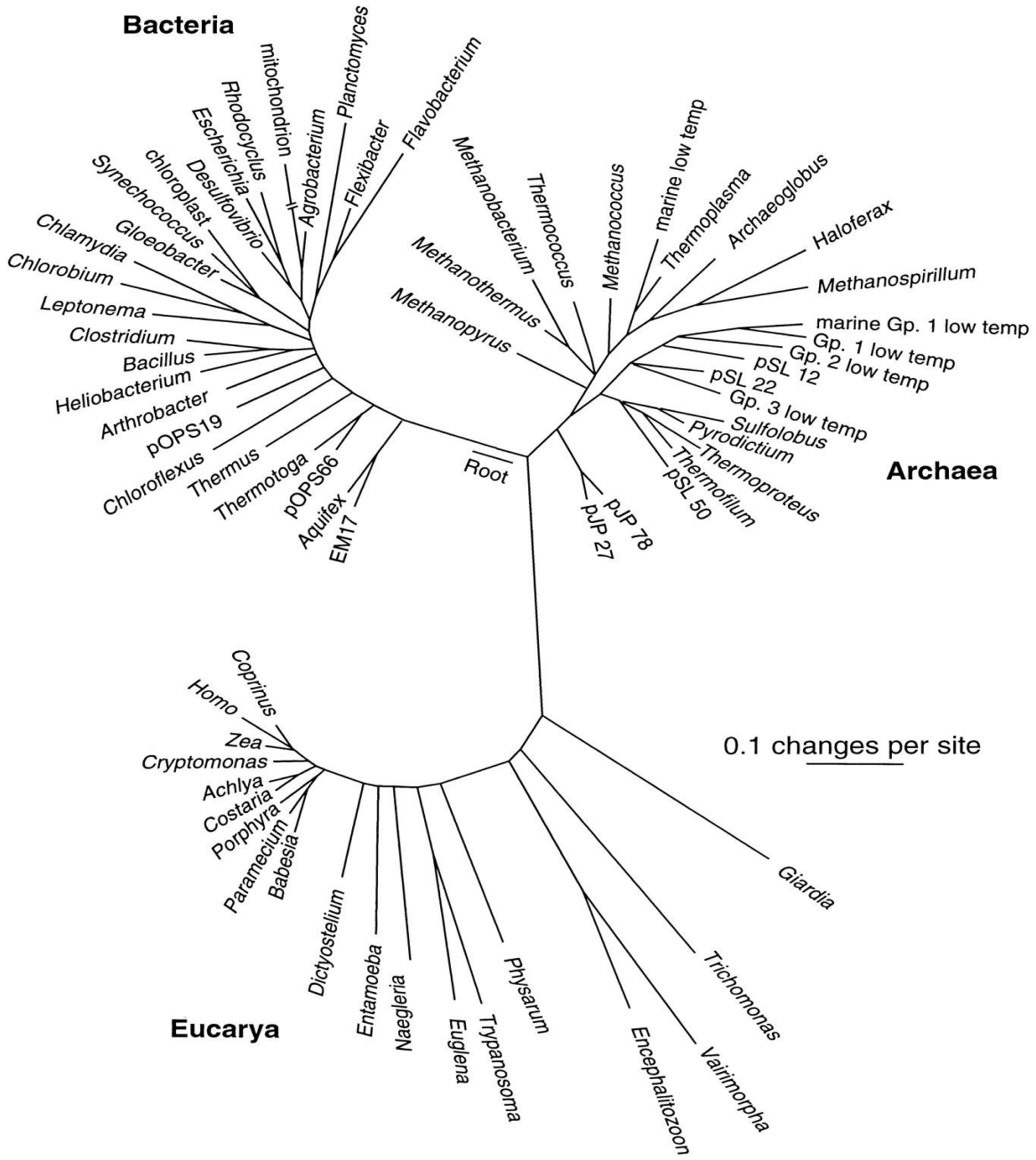


Figure 1.1: Universal phylogenetic tree based on 16S rRNA sequences. Reproduced from Pace (1997) with kind permission of Professor Norman Pace (University of Colorado, USA). The scale bar corresponds to 0.1 changes per nucleotide.

The serovars are classified into two biovars: trachoma and lymphogranuloma venereum (Manavi, 2006). Serotypes A-C are related to ocular disease, serotypes D-K with ophthalmic and genital infections and L_1-L_3 with LGV (Batteiger, 1996; Manavi, 2006). There is evidence from a number of studies that show serovars D, E, F are usually a more frequent cause of genital infection (Manavi, 2006). Interestingly, there is a suggestion that some serovars or serovar variants might be more strongly related with disease than others (Manavi, 2006). *C. trachomatis* serotyping showed the particular association of some serovars with ocular and urogenital infections although for serovars B was shown overlapping for both ocular and genital infections (Wang and Grayston, 1970; Jalal *et al*., 2007). However, there is controversy and further work is required to investigate these claims.

The unique life cycle or developmental cycle of *C. trachomatic* includes two distinct morphological forms of the microorganism: the intracellular reticulate bodies and the extracellular (infective) form called elementary bodies (Wolner-Hanssen and Mardh 1984; Black, 1997). Attachment of EBs to host cells is the most important event in chlamydial pathogenesis (Zhang and Stephens, 1992; Fawaz *et al*., 1997; Stamm, 1999). The possible mechanism of infection (Taraktchoglou *et al*., 2001) is using host cell heparin sulfate and the putative mechanism of attachment to cells such as sperm is through *C. trachomatis* lipopolysaccharide (Hosseinzadeh *et al*., 2000; Eley *et al*., 2005a; 2005b) and it determines entry of chlamydia into target cells and formation of inclusions or vacuoles. EBs are protected from host cellular defence mechanism by this unique process (Zhang and Stephens, 1992; Fawaz *et al*., 1997; Stamm, 1999).

The developmental cycle of *C. trachomatis* is unique in microbiology (see Figure 1.2). It is characterised by a small (approximately 0.3µm in diameter) infectious elementary body (EB) and a larger (1µm) dividing intracellular reticulate body (RB).

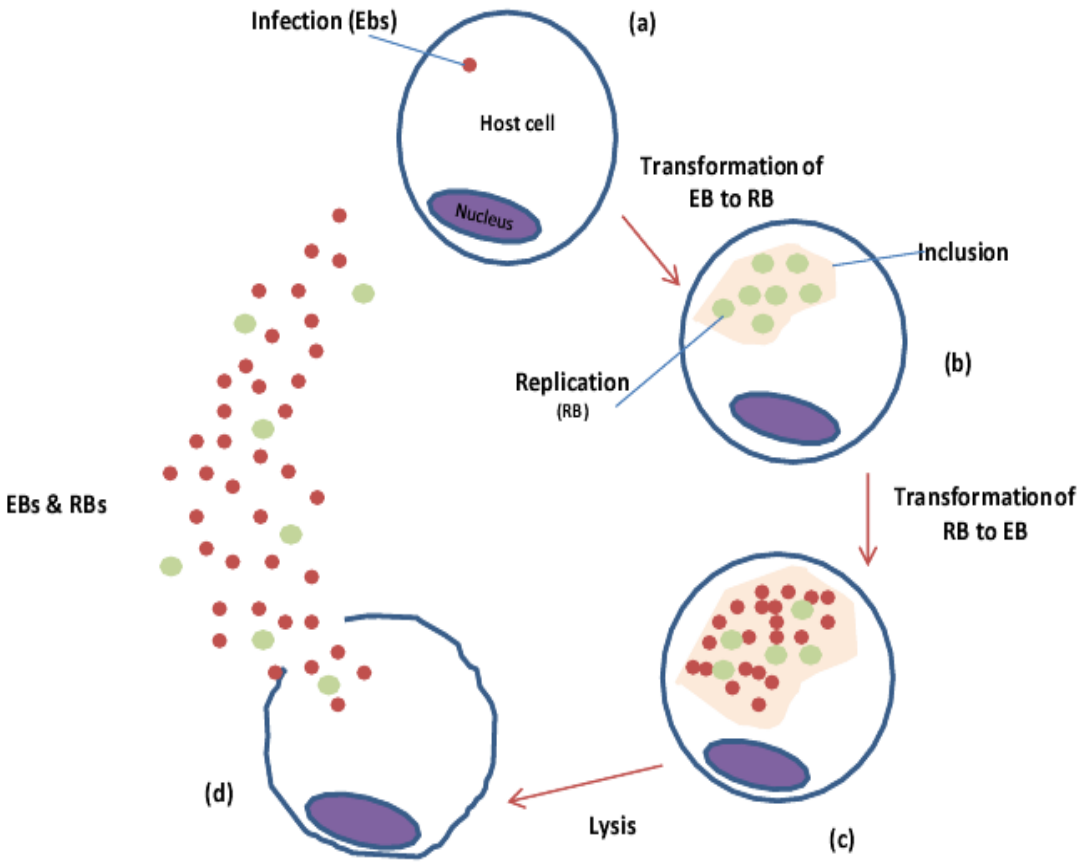


Figure 1.2: Developmental cycle of *C. trachomatis*. EBs are responsible for attachment to host cells, so the first step in the infection process is attachment of EBs to mucosal epithelial surfaces of host cells (a), then the EB is endocytosed into the host cell and a vacuole develops in the cytoplasm (b). Two hours post infection, EBs change to RBs. Then replication of RBs through binary fission occurs. Over 8-12 hours, the RBs increase in size and volume, and then an inclusion membrane is formed (c). Afterwards the inclusion bodies are expanded and at 18 hours post infection, RBs are differentiated to EBs. Finally, EBs are released from the infected host often by lysis of the host cell (d). The time of lysis and release of EBs are dependent on serovars of chlamydia. These events take place 36 to 72 hours post infection (Paavonen & Eggert-Kruse, 1999).

1.2.3 Effect of *C. trachomatis* infection in men

C. trachomatis, as a cause of the most prevalent STD in the world, has been studied more with regard to its effect on the female genital tract. Moreover, the clinical effect of infection is thought to be greater in the female compared with male infections. To investigate the effect of *C. trachomatis* infection in male patients, more studies have been focused on its effect on semen parameters rather than direct effects on the male reproductive tract (Keck *et al*., 1998; Norman, 2002). There is controversy about relationship between *C. trachomatis* infection and semen quality: some studies have shown that infection is associated with poor semen quality (Witkin *et al*., 1995; Cengiz *et al*., 1997) and some of them have claimed this is not (Eggert-Kruse *et al*., 1990; Hosseinzadeh *et al*., 2004). However, because the methodology for *C. trachomatis* detection and semen analysis techniques are not comparable (Pacey and Eley, 2004) it is difficult to make comparisons between studies.

Direct exposure to *C. trachomatis* can affect the function of human spermatozoa (Eley *et al*., 2005b) and in infected individuals might contribute in subfertility by an independent route of any damage to the reproductive epithelium (Pacey and Eley, 2004). Although most infected men are asymptomatic, urethritis is the most prevalent symptom (Eley *et al*., 1992). In younger men epididymitis is often seen with *C. trachomatis* infection (Eley *et al*., 1992). Epithelial damage of *C. trachomatis* infection can reduce spermatogenesis and induce immunological responses that destroy sperm (Gonzales *et al*., 2004). According to Wan *et al*., (2003) and Eley *et al*., (2005) *C. trachomatis* infection can cause sperm DNA fragmentation because sperm apoptosis is triggered. Lipopolysaccharide (LPS) extracted from *C. trachomatis* elementary bodies (EBs) cause apoptosis by a caspase-mediated mechanism (Hosseinzadeh *et al*., 2001). Also sperm motility and viability decrease when live *C. trachomatis* EBs are incubated with spermatozoa from normal men. LPS extracted from *C. trachomatis* EBs had the same effect (Hosseinzadeh *et al*., 2001; 2003). In a different study, *C. trachomatis* serovar E was shown to cause sperm death within a few hours of exposure, and also stimulated the tyrosine phosphorylation of 2 major sperm epitopes (Hosseinzadeh *et al*., 2000). Two toxic components of LPS including lipid A and 3-deoxy-D-manno-octulosonic acid have also shown an apoptosis effect similar to LPS by a caspase-mediated mechanism (Hakimi *et al*., 2006). High levels of sperm DNA fragmentation

are seen in infected patients and decreases after antibiotic therapy (Satta *et al*., 2006; Gallegos *et al.*, 2008).

All these effects are probably mediated by the attachment of EBs to sperm. Wolner-Hansen and Mardh (1984) were the first to report attachment of *C. trachomatis* to human spermatozoa *in vitro* using fluorescence and transmission electron microscopy. They reported that the number of EBs attached to spermatozoa depends on the concentration of EBs in which with the spermatozoa were incubated. Mavrov (1995) reported the attachment of *C. trachomatis* to different regions of spermatozoa as seen by transmission electron microscopy, and there is also one report analyzing patient spermatozoa following chlamydial infection *in vivo* (Erbengi, 1993). However, none of these results have fully defined the attachment of *C. trachomatis* to spermatozoa at the molecular level.

The presence of bacteria inside of seminal leucocytes indicates that infection can be persistent and lead to a chronic phase of infection (Gallegos-Avila *et al*., 2009). In infertile men higher pH and seminal leucocytes as well as lower percentage progressive motile spermatozoa were associated with chlamydial infection (Hosseinzadeh *et al*., 2004; Kokab *et al*., 2010). High seminal plasma IL_6 and IL_8 were associated with *C. trachomatis* infection (Eggert-Kruse *et al*., 2001; Kokab *et al*., 2010).

C. trachomatis IgG antibody is known as a marker of previous or persistent infection (Mol *et al*., 1997). Immunoglobulin G antibody to *C. trachomatis* in the male partner is related to presence of IgG antibody in the female partners and decreased pregnancy rate (Idahl *et al.*, 2004). There were no differences in outcome of pregnancy (either spontaneously or related treatment) between IgG positive and IgG negative groups (Idahl *et al.,* 2004). Also Idahl *et al*., (2007) found that *C. trachomatis* IgA antibody in infertile men was correlated with reduced sperm motility and pregnancy rates. IgA in combination with IgG was associated with decreased in sperm concentration, the number of progressive spermatozoa, and increasing in the teratozoospermia index. A high percentage of dead spermatozoa and high prevalence of leucocytes in semen were seen in IgA positive men (Idahl *et al*., 2007).

1.2.4 Clinical appearances of *C. trachomatis* infection in men

1.2.4.1 Urethritis

Although chlamydial genital tract infections are asymptomatic in approximately 50% men (Taylor and Haggerty, 2011) non-gonococcal urethritis (NGU) is the most common clinical syndrome in chlamydial infection. This was established by Holms *et al*., (1975). Urethritis following chlamydial infection may lead to prostatitis, vesiculitis or epididymitis (Gonzales *et al*., 2004). Post gonococcal urethritis (PGU) refers to a persistent non-gonococcal urethritis following treatment for gonococcal urethritis. *C. trachomatis* should be considered in treatment of PGU because it is responsible for 70-80% of PGU as well (Schachter, 1999).

1.2.4.2 Prostatitis

C. trachomatis has been detected in the seminal vesicles and prostate and it is suggested that it may affect their function (Bornman *et al*., 1998). High prevalence of chlamydia was reported in the male with prostatovesiculitis (Soffer *et al*., 1990). According to Weinder *et al*., (1991), one-third of unspecific accessory gland infection is due to chlamydial prostate-vesiculitis. In prostatitis patients *C. trachomatis* has been detected in up to 30% of prostatic or semen secretion samples and in 2.2% to 33% of prostate tissue samples (Taylor and Haggerty, 2011). Young male fertility has been affected by *C. trachomatis* infection in chronic prostatitis patients and recovery of semen quality has not always been seen in *C. trachomatis* infection after antibiotic therapy (Cai *et al*., 2011).

1.2.4.3 Epididymitis

Many studies have reported that STD-associated infections in the male can lead to occlusions in the genital tract, which in the acute phase via the spread of microorganisms from the urethra, can cause damage to tubular tracts and to the cells involved in spermatogenesis (Berger, 1990; Villegas *et al*, 1991). *C. trachomatis* is a cause of idiopathic epididymitis, and Moller and Mardh (1989) provoked experimentally *C. trachomatis* epididymitis in a male monkey model. When orchitis is seen with epididymitis, decreasing sperm production and testicular atrophy is observed (Berger, 1990). Approximately 45%–85% of men with epididymitis have had a prior *C. trachomatis* infections and/or gonococcal infections (Taylor and Haggerty, 2011).

1.2.5 Effect of *C. trachomatis* infection in women

Chlamydial infections are predominantly asymptomatic in women and can disseminate through sexual contact (Pacey and Eley, 2004). Although *C. trachomatis* is a main cause of cervicitis, in half of the cases it is seen with urethritis (Paavonen and Eggert-Kruse, 1999). Both the lower and upper genital tract can be involved in this infection (Paavonen and Eggert-Kruse, 1999). Furthermore, asymptomatic infection may lead to severe complications such as PID, salpingitis, chorioamnionitis, ectopic pregnancy, premature delivery, neonatal infections and infertility (Paavonen and Eggert-Kruse, 1999; Gonzales *et al*., 2004). Generally C. *trachomatis* infection in women has more visible effects or complications.

Tubal factor infertility as a result of PID has been seen in women who have never been diagnosed because infection is silent and there is no history of PID (Gonzales *et al*., 2004). Subclinical PID is the cause of a greater proportion of PID-related sequelae (Sweet, 2011) and increasing *C. trachomatis* infection as a cause of clinical and subclinical PID is a concern. Approximately 20% of women with lower genital tract infection due to *C. trachomatis* will develop PID, 4% will develop chronic pelvic pain, 3% infertility, and 2% adverse pregnancy outcome (Paavonen and Eggert-Kruse, 1999). This is why chlamydial infections are considered as primarily a woman's health care issue (Paavonen and Eggert-Kruse, 1999). The most important preventable cause of infertility and adverse pregnancy outcome is PID following chlamydial infection (Westrom *et al*, 1994).

There are some studies that have verified a strong relationship between serum antibodies (Ab) to *C. trachomatis* and tubal factor infertility or ectopic pregnancy both in women with or without history of PID. These studies suggested that chlamydial infection might be a cause of a large percentage of tubal factor infertility and ectopic pregnancy (Cates *et al*., 1991). Ectopic pregnancy in the first trimester of pregnancy is the main cause of maternal mortality in developing countries. In comparison with women with no history of PID, women with a history of PID have a 7 to 10 fold increased risk of tubal pregnancy (Westrom *et al*, 1981; Westrom, 1994).

According to a research by Eggert-Kruse and colleagues, titres of antichlamydia immunoglobulin were elevated in subfertile couples and these antibodies (Ab) are more frequent in the women who had seropositive partners (Eggert-Kruse *et al*., 1997). Punnonen *et al*., (1979) were the first to study the link between tubal factor and past chlamydial infection and they found that patients with normal hysterosalpingography had significantly lower titres of antichlamydia Abs compared with those that had bilateral tubal obstruction. Obstructed Fallopian tube is four times more likely to be found in women with previous chlamydial infection (Gonzales *et al.*, 2004).

Some studies reported *C. trachomatis* may also contribute to pregnancy complications, including premature rupture of membranes, preterm birth, low birth weight and still birth (Gravett *et al.*, 1986; Hollegaard *et al*., 2007). Asymptomatic *C. trachomatis* infections are thought to induce early pregnancy loss through the stimulation of immune mechanisms (Witkin *et al*., 1998).

There is an association between *C. trachomatis* infection and pregnancy loss especially in the first-trimester. This is explained in two ways: (1) direct infection of the oocyte or zygote or the immune response to heat shock proteins expressed by the zygote that is triggered by a previous *C. trachomatis* infection (Vigil *et al*, 2002); and (2) stimulation of toll-like receptor 4 (TLR4) *in vitro* by chlamydial heat shock protein 60 antigen that is responsible for inducing apoptosis in primary human trophoblast, placental fibroblast and human syncytiotrophoblast cell lines (Equils *et al*., 2006). This may help understand the molecular mechanism of pathogenesis for poor pregnancy outcome in women with persistent chlamydial infection.

In the host defence mechanism against *C. trachomatis* genital tract infections it seems that toll-like receptors (TLRs) play a role, because some of them are expressed in epithelial cells in the human genital tract and are able to recognise *C. trachomatis* pathogen-associated molecular patterns (PAMPs). Measuring *C. trachomatis* IgG antibodies in serum has been introduced in the fertility work-up and is nowadays recommended to assess the risk of tubal pathology in subfertile women in an inexpensive and non-invasive way (den Hartog *et al.*, 2006; Akande *et al*., 2010).

1.2.6 Clinical appearances of *C. trachomatis* infection in women

1.2.6.1 Lower genital tract infection

The cervix and urethra are the main location of *C. trachomatis* infection that can lead to cervicitis and urethritis respectively. Mucopurulent discharge (37%) and hypertrophic cervical ectopy (19%) have been reported in the infected women. Abdominal pain, inter-menstrual bleeding can be seen as well. Serious complications (e.g. tubal factor infertility, ectopic pregnancy and pregnancy loss) have been seen after ascending *C. trachomatis* infection to the upper genital tract (Stamm *et al*., 1980; Stamm, 2008; Taylor and Haggerty, 2011).

1.2.6.2 Pelvic Inflammatory Disease (PID)

Acute PID is a major complication of genital *C. trachomatis* infection (Gonzales *et al*., 2004; Taylor and Haggerty, 2011) that is isolated in 25% of patients. However progression rates vary widely. Studies have shown that 2.0% - 4.5% of genital chlamydial infection developed clinical PID during 14 days among untreated women (Morre *et al*., 2002; Geisler *et al*., 2008). Also in longer follow-up studies the rates 0% - 9.5% PID have been reported among untreated women (Bakken *et al*., 2009; Oakeshott *et al*., 2010). In Norway, the largest study conducted (1990-2005) showed 1.09% (48/4413) of cases that tested positive for *C. trachomatis* were hospitalized for PID (Bakken *et al*., 2009). The magnitude of PID is due to its subsequent sequelae including chronic pelvic pain, ectopic pregnancy, and infertility (Brunham *et al*., 1988; Chow *et al.*, 1990; Westrom *et al.*, 1992).

1.2.7 Pathogenesis of chlamydial infections

1.2.7.1 Epidemiology

Chlamydiae cause a wide variety of disease. The highest infection rates are found among the young and the poor (Schachter, 1999). Because women are more likely to be screened the reported rates in females are higher than the reported rates in males (Schachter, 1999). Prevalence of *C. pneumoniae* in adults is about 70% and infects a majority of humans as an important respiratory pathogen (Grayston *et al*., 1986; Schachter, 1999). *C. psittaci* is a common pathogen of avian species that are the source for human disease as they act as potential reservoirs. Moreover, *C. psittaci* can infect

mammals (Schachter, 1999). Trachoma is still a major cause of blindness in hyperendemic areas (tropical and subtropical countries) in 2nd and 3rd year of life. However its blindness is preventable (Thylefors *et al*., 1995; Schachter, 1999).

1.2.7.2 Immunity in chlamydial infections

As the genital tract, respiratory system and eyes may be involved in chlamydial infections, the immune responses depend on the variety of tissues that are involved in infection and their response. Humoral and cell mediate immune responses are involved in chlamydial infections (Paavonen and Eggert-Kruse, 1999).

1.2.7.2.1 Innate defence mechanisms

Researchers have more recently focused on the role of innate immune mechanism in the early response to *C. trachomatis* infection (Tiitinen *et al*., 2006; Taylor *et al*., 2012). The involvement of Toll-like receptors (TLRs) has been investigated (den Hartog *et al*., 2006; Equlis *et al*., 2006; Taylor *et al*., 2012) and 4 TLR genes (TLR_1, TLR_2, TLR_4, and TLR_6) were associated with *C. trachomatis* infection among women with clinically suspected PID (Taylor *et al*., 2012). TLRs act as pattern recognition receptors to enable cells to identify pathogen-associated molecular patterns (Beatty *et al*., 1993). The most important factor in host defence against *C. trachomatis* infection is interferon γ (INF γ) a typical product of T helper cells (Th_1). Expression of the IL-10 marker of Th_2 cell activation is related to disease susceptibility (Beatty *et al*., 1993). Different pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, and TNFα were seen *in vitro* and *in vivo* studies of epithelial cells infected with *C. trachomatis* (Mpiga *et al*., 2006; Al-Mously and Eley, 2007).

1.2.7.2.2 Humoral response

IgA is considered a critical protection factor in epithelial cells. In chlamydial infection of the genital epithelium IgG and IgM have been detected in serum, and both IgA and IgG have been measured in secretions (Agrawal *et al*., 2007). Patients with both ocular and genital chlamydial infection have a higher level of IgG in serum compared with patients who have genital or ocular infection (Patel *et al*., 1992). In a study of primary and recurrent genital chlamydial infections, cervical IgA and IgG were higher in primary infection than recurrent infection (Agrawal *et al*., 2007). IgG antibody is detectable more than two years after infection, its level dramatically decreased 50 days

after infection, whilst IgA levels are detectable up to 100 days (Batteiger and Rank, 1987). Eggert-Kruse *et al*., (2011) showed IgG, IgM and IgA antichlamydial antibodies in serum were interrelated and associated with IgA in seminal plasma (Eggert-Kruse *et al*., 2011).

1.2.7.2.3 Cell mediated immunity (CMI)

CMI plays a key role in defence against intracellular pathogens. In the murine model, localized *C. trachomatis* genital infection is mediated by chlamydial- specific CD_4 cells (Ramsey *et al*., 1991). CD_4^+ cells play a significant role in adaptive immunity to *C. trachomatis* infection (Ramsey *et al*., 1991; Su and Caldwell, 1995). Studies have shown the role of CD_4^+ and CD_8^+ cells in chlamydial infection that lack of these cells has been observed (Gervassi *et al*., 2004b; Marks *et al*., 2007). Although the role of a CMI response has been shown in animal models (murine, guinea pig, primate) the important role in genital infection by *C. trachomatis* has been observed in primates for CD_8 cells (Van Voorhis *et al*., 1996). Kelly and Rank (1997) investigated the mechanism of CD_4 cells during a natural infection and their results supported the role of CD_4 cells to protect against vaginal *C. trachomatis* in mouse, also CD_8 lines and clones protect in the mouse pneumonitis biovar of *C. trachomatis* (MoPn).

In women without inflammation in the genitalia, the CD_8 cells were predominantly intraepithelial lymphocytes whereas increasing numbers of intraepithelial CD_8, CD_4 lymphocytes and antigen presenting cells were seen in women with cervicitis (Pudney *et al*., 2005). Wyrick (1999) stated that a CMI response has been observed in both trachoma patients and in animal models. In viral, parasitic and bacterial infections, NK T cells have a role in host defence (Wyrick *et al*., 1999).

1.2.8 Laboratory methods for detecting *C. trachomatis*

1.2.8.1 Cell culture

This was performed in the early days of *C. trachomatis* screening by inoculation of specimens such as urethral swabs onto monolayer cell cultures (Mardh *et al*., 1980). Chlamydial inclusions can be observed in the monolayer 48-72 h post infection by different staining methods according to the presence of sufficient viable micro-

organisms in the swab. The specificity of the test is 100% to detect *C. trachomatis* in urogenital specimens whilst sensitivity is about 70-80% (Lee *et al*., 1995; Black, 1997; Carder *et al*., 2006). Although in clinical specimens McCoy is the most common cell line used to grow *C. trachomatis* (Ehert and Judson, 1989) many cell lines including Hela-229, BHK-21, Hec1B and green monkey kidney cells have been used (Kuo *et al*., 1972; Stamm *et al*., 1983). After using non-invasive samples such as urine to detect *C. trachomatis* and discovering that semen components are toxic to growth and maintenance of the monolayer, it was determined that cell culture was too insensitive and the method was abandoned (Taylor-Robinson and Thomas, 1991).

1.2.8.2 Direct Immuno Fluorescence (DIF) staining

DIF has been used to detect *C. trachomatis* in clinical samples. Although the number of elementary bodies is a factor to define a positive sample (Tilton *et al*., 1988; Thomas *et al*., 1993) the technique is based on the ability to visualize chlamydial particles or inclusions by microscopy. Monoclonal antibodies against MOMP of *C. trachomatis*, in both clinical and laboratory strains, produce a specific fluorescent brightness of EBs in this technique (Cles *et al*., 1988). Advantages of the test in comparison with tissue culture and EIA (see below; section 1.2.8.4) are its simplicity and rapidity. The specificity of the test is 82% to 100% and sensitivity 68% to 100% (Black, 1997). The test is often applied as a confirmation test for positive results of non-culture tests (Chan *et al*., 1994).

1.2.8.3 Other chlamydial staining methods

To detect chlamydial inclusions, Gram, Giemsa or Iodine staining have been traditionally used. Neonatal inclusion conjunctivitis is diagnosed by Giemsa staining due to the simplicity of the test (Black, 1997). However, the test is not used commonly because of low sensitivity and specificity compare with DIF (see section 1.2.8.2). Since staining artifacts can be considered as chlamydial inclusions, the reading of the stained slides needs to be performed by expert staff (Schoenwald *et al*., 1988). Due to the variable results, Gram staining has little practical value, although chlamydia is considered a Gram negative micro-organism. Iodine staining is a selective stain of glycogen, therefore is not able to distinguish infections in the cells which normally contain glycogen and has low sensitivity in detecting the inclusions (Black, 1997).

1.2.8.4 Serological tests

An approach to detect *C. trachomatis* in clinical specimens is the identification of antibodies to chlamydia. In general, the use of chlamydial serology to detect *C. trachomatis* has several limitations. There is no single sensitive and specific *C. trachomatis* antibody test (Johnson & Horner, 2008). Also, there is often cross-reactivity with *pneumoniae* that cause variable specificity and sensitivity. Infections are long lived, thus a positive antibody test cannot distinguish between a current and a previous infection (Black, 1997; Johnson & Horner, 2008). The tests which have been developed for detection of antibody to chlamydial infections include:

i) Micro-Immuno Fluorescence (MIF) which is considered a gold standard for chlamydia species and serovars among the serological tests (Wang and Graystone, 1970; Bax *et al*., 2003).

ii) Complement Fixation Test (CFT) which is the most common test used for diagnosis of chlamydial infections by recognising the genus-specific LPS antigen via complement-fixing antibodies. It is not specific for any chlamydial species (Black, 1997).

iii) Enzyme Immuno Assays (EIAs) that detect the immune response to chlamydial genus-specific LPS of EBs or RBs. The test has cross-reactivity with *C. pneumoniae, Bacteroides species* and *Escherichia coli* due to high prevalence of these pathogens (Black, 1997; Eggert-Kruse *et al*., 1995; Ivanov *et al*., 2009). Therefore the sensitivity is poor compared with molecular methods. Recently a new assay that showed no cross-reactivity with *C. pneumoniae* has been developed (Wills *et al*., 2009).

1.2.8.5 Molecular methods

Using *in situ* hybridization (ISH) in clinical samples was an early method to detect *C. trachomatis* DNA. However, the test was not sensitive enough and when nucleic acid amplification testing (NAAT) became available, ISH was abandoned (Yoshida *et al*., 1998). NAATs are the most sensitive test for detection of chlamydial infections (Schachter, 2001; Schachter *et al*., 2006).

Polymerase chain reaction (PCR) and ligase chain reaction (LCR) have better sensitivities than non-molecular methods also these are more effective in detecting

chlamydial infection in asymptomatic and low prevalence populations (Watson *et al*., 2002; Skidmore *et al*., 2006). The tests have sensitivities of greater than 90% and specificities approaching 100%. Since NAATs detect nucleic acid targets, their advantage is that they do not need viable micro-organisms (Black, 1997). LCR was a commercial system in the 1990s, and has been used to detect *C. trachomatis* in semen (Eggert-Kruse *et al*., 1997; 2002a,b; Hosseinzadeh *et al*., 2004) but is no longer available.

The first detection of *C. trachomatis* in semen samples using PCR was in 1993 (Van den Brule *et al*., 1993). Afterwards a number of other studies also used it (Ochsendorf *et al*., 1999; Hosseinzadeh *et al*., 2004; Gdoura *et al*., 2008). A more recent development has been the introduction of real-time or quantitative PCR, that detect *C. trachomatis* DNA copy numbers (Al-Mously *et al*., 2009). Amplification and quantification of the target nucleic acid take place in a single step and closed system (Jalal *et al*., 2007). Therefore, the advantage of this method includes high sensitivity and specificity, quantitative assay, providing faster result than gel-based PCR and avoiding contamination (Alexander *et al*., 2007; Jalal *et al*., 2007a).

1.3 History of *Mycoplasma genitalium*

M. genitalium is not well studied. The first time *M. genitalium* was isolated by culture was in 1980 when it was isolated from two of 13 men with non-gonococcal urethritis (Tully *et al*., 1981). However, in spite of several efforts the organism has not been isolated by culture since that time (Jensen *et al*., 1993; Fraser, 1995) and in 1999 it was again performed by Luo *et al*., (1999) successfully. *M. genitalium* is considered as a cause of non-gonococcal urethritis (NGU) in both males and females, although it is difficult to isolate from clinical samples, and cross reactivity with *M. pneumoniae* has hampered the development and use of serologic tests (Jensen *et al*., 1993).

1.3.1 Taxonomy and classification of *M. genitalium*

M. genitalium is a small bacterium which is a member of the family *Mycoplasmatacae* and *Mollicutes* class (Fraser, 1995). *Mycoplasma*s live on the ciliated epithelium of the primate genital and respiratory tracts (Fraser, 1995; Peterson, 1995). *M. genitalium*, after the endosymbiont *Carsonella ruddii*, is the second-smallest bacterium and the smallest known free-living bacterium (Fraser, 1995; Peterson, 1995). It was also

considered to be the organism with the smallest genome before the discovery of *Nanoarchaeum equitans* in 2002 (Huber *et al*., 2002; Westers *et al*., 2003).

The genome of *M. genitalium* consists of 521 genes (428 protein encoding genes) in one circular chromosome and was studied by Peterson in 1993 with random sequencing (Peterson, 1993; 1995; Fraser, 1995). It was the second complete bacterial genome ever sequenced, after *Haemophilus influenzae*. Therefore, these characteristic of *M. genitalium* made it the organism of choice in the Minimal Genome Project (Glass *et al*., 2006). In humans several mycoplasma species have been isolated. For seven of them, the genital tract is the main site of colonization: *M. hominis*, *M. genitalium*, *M. fermentans*, *M. penetrans*, *M. pneumoniae*, *M. primatum* and *M. spermatophilum* (Uuskula, 2002; Patel *et al*., 2010).

1.3.2 Cell structure

The lack of a cell wall is typical of mycoplasma species and *M. genitalium* only has a plasma membrane (Razin, 1998). Neutral lipids, phospholipids and glycolipids are located in the cell membrane. *M. genitalium* as a motile mycoplasma connects to surfaces using a complex terminal attachment organelle known as specific tip structure and moves across the tip (Razin, 1998). Tip construction causes *M. genitalium* to attach to eukaryotic cells and the adhesin protein (MgPa) is responsible for attachment to cells (Peterson 1995; Razin, 1998). Invasion of mycoplasmas into the host cell cytoplasm affects cell function and eventually results in cell disruption and necrosis (Uuskula, 2002).

Many biosynthetic pathway mechanisms are lacking in *M. genitalium* due to a lack in many genes coding for them. However, *M. genitalium* needs only small amounts of ATP for its limited biosynthetic pathways and therefore it grows well *in vivo* (Razin, 1998). To produce ATP, *M. genitalium* depends on glycolysis, which is less efficient than oxidative phosphorylation. There are no quinones and cytochromes in any of the mycoplasmas and also there are no genes for synthesizing any fatty acids; therefore, they use their host fatty acids (Razin, 1998).

1.3.3 Effect of *M. genitalium* infection in men

M. genitalium is considered as a cause of inflammatory lower genital tract syndrome (McGowin, 2011). Majority of patients with genital tract infections are not symptomatic and asymptomatic infections of the male urogenital tract are difficult to detect because taking samples are unacceptable to many asymptomatic men (Mardh *et al*., 1981, Gdoura *et al*., 2008). The impact of *M. genitalium* colonization of the genital tract on semen parameters and male fertility remains unclear and *M. genitalium* has seldom been investigated in the semen of infertile men. Gdoura *et al*., (2007) investigated the frequency of *M. genitalium* in semen sample of infertile men by PCR and found 5% positivity, as well as a very high concordance between detection of mycoplasma DNA in semen and first void urine. By comparison, Kjaergaard *et al*., (1997) showed 0.9% positivity by culture and this difference could be because the two studies used different methods for detection. In some studies there is a discrepancy between the detection of mycoplasma DNA from semen and corresponding FVU specimens which may indicate an asymptomatic infection in the seminal vesicles and the epididymis or urethra (Tully *et al*., 1981). Wang *et al*., (1997) stated that during ejaculation semen might be contaminated by genital Ureaplasma (*U. urealyticum* and *U. parvum*) and genital mycoplasmas (*M. genitalium* and *M. hominis*) as they are natural inhabitants of the male urethra. In susceptible populations genital opportunistic organisms including *M. hominis* and *Ureaplasma* species are related to invasive genitourinary infections (Jensen *et al*., 1991).

Spermatozoa have been suggested as vectors for bacterial spread from the male to the female genital tract and *in vitro* experiments have revealed *U. urealyticum*, *N. gonorrhoeae, Escherichia coli* and *C. trachomatis* and *Mycoplasma* (*hominis* and *genitalium*) attach to human spermatozoa (Friberg and Fullan, 1983). Taylor-Robinson and Manchee (1967) reported non genital mycoplasma (e.g. *M. pneumoniae*) could bind human spermatozoa. *M. genitalium* is able to attach to many different cell types including: erythrocytes, cells of the Fallopian tube, Vero cells, airway cells, HEp-2 cells, and even plastic (Morrison-Plummer *et al.,* 1987; Collier *et al*., 1990; Jensen *et al*., 1994; Baseman *et al*., 1996). The attachment of *M. genitalium* to spermatozoa has been reported by Svenstrup (2003) and it has been noted that this did not cause any morphological change (Svenstrup, 2003; Baczynska *et al.,* 2007). The preferred site of

attachment was the sperm midpiece/neck. There is a suggestion that the host cell receptor for *M. genitalium* is probably a sialoglycoconjugate (Svenstrup, 2003). Also Czuppon (1984) stated spermatozoa receptors, which could function as receptors for *M. genitalium,* expose sialoglycoproteins at their surface (Svenstrup, 2003). As shown by x-ray microscopy, the attachment of *M.genitalium* to spermatozoa seemed to be mediated by tips (see section 1.3.2). Further experiments are needed in order to find whether the attachment is specific (Svenstrup, 2003).

The effects of genital mycoplasmas on semen parameters are controversial. Andrade-Rocha (2003) and Soffer *et al*., (1990) showed that there was no effect on semen quality; Pannekoek *et al*., (2000) showed that low sperm concentration was only seen in the male partner of infertile couples with mixed infection (mycoplasmas & ureaplasmas). Gdoura *et al.,* (2007) stated the ability of mycoplasma to attach to spermatozoa can affect directly via cellular interactions that lead to influence on semen parameters (Gdoura *et al.,* 2007). Sperm concentration has negative correlation with *M. genitalium* infection (Gdoura *et al*., 2007). Since there was no significant association between mycoplasma infection and semen leukocytes, the absence of leukocytospermia does not exclude the presence of genital mycoplasmas (Pannekoek *et al*., 2000). Samra *et al*., (1993) investigated the prevalence of *C. trachomatis* and *M. hominis* among infertile couples and found the prevalence of *M. hominis* was significantly higher in the infertile men and women (Samra *et al*., 1993). Its role on fertility can be cause of urethritis and also can be sexually transmitted with rates similar to *C. trachomatis* that lead to female partner infection as well (Jensen *et al*., 2006).

1.3.4 Clinical appearances of *M. genitalium* infection in men

Urethritis is the most common symptom in men although most cases are asymptomatic (Jensen *et al.,* 1993). In men with non-gonococcal urethritis (NGU), the *M. genitalium* incidence is considerably higher than those who are without symptoms (Jensen *et al.,* 1993). *M. genitalium* has been isolated from men with NGU and without urethritis, and also was isolated from rectal swabs from both heterosexual and homosexual men (Taylor-Robinson *et al.,* 1985). Also data from a study of non-chlamydial non-gonococcal urethritis (NCNGU) patients show a higher number of *M. genitalium* DNA copies in first void urine (FVU) than from asymptomatic men

(Yoshida *et al*., 2002). Unlike the situation for *C. trachomatis*, there are no studies suggesting a role for *M. genitalium* in prostatitis and epididymitis.

1.3.5 Effect of *M. genitalium* infection in women

Although the bacterium has been identified in urethral and cervical samples, the role of *M. genitalium* in women has been less well studied in comparison to men (Jensen *et al.*, 1991; Palmer *et al.*, 1991). Moreover, in comparison with *C. trachomatis*, the role of *M. genitalium* in female infertility is less well studied and its role on human infertility is still not clear. Although *N. gonorrhoeae* and *C. trachomatis* are the most common agents of PID, some studies suggested that infertility is more common in non-gonococcal infections such as *M. genitalium* (Westrom, 1975; Eggert-Kruse *et al*, 1997). *M. genitalium* has been implicated in infertility due to compromised function of the uterine tubes, and the role of *M. genitalium* in non-gonococcal PID (Clausen *et al*., 2001) and PID is a cause of several major complications including chronic pelvic pain, tubal factor infertility and ectopic pregnancy (Westrom, 1975). New studies will be necessary that focus on the dynamic development of diagnostic procedures, especially the more common use of molecular methods, in discovering the role of mycoplasmas in female infertility. Antibodies to MgPa (adhesin protein) were found in the patients with TFI to investigate the role of *M. genitalium* in infertility. The study indicated that uterine tube function was compromised and led to infertility (Uno *et al.*, 1997).

Clausen *et al*., (2001) found antibodies to *M. genitalium* in 22% of their patients with TFI. Haggerty *et al*., (2006) reported prevalence of *M. genitalium* among women with non-gonococcal non-chlamydial PID. They found overall 14% positivity by PCR among an urban US population that was similar to PCR investigations in Kenyan women (Cohen, 2002) with endometritis (16%), and United Kingdom women (Simms, 2003) with clinically suspected PID (13%). Sera from TFI patients were positive to MgPa (adhesin protein) that indicates capacity of *M. genitalium* to create an infection independently of *C. trachomatis* (Clausen *et al*., 2001).

During pregnancy, *M. genitalium* infection can lead to chorioamnionitis and further pregnancy complications (Jurstrand *et al*., 2007). *M. genitalium* had been transmitted from mother to the newborn, but the magnitude of this finding is vague. There is no statistical correlation between PID or EP and *M. genitalium* antibodies (Jurstrand *et al*.,

2007). When younger individuals were investigated for an association between PID or EP and *M. genitalium* antibodies, a slight association was found using the lipid-associated membrane protein-enzyme immunoassay (Jurstrand *et al*., 2007). Also other adverse pregnancy outcomes have been investigated including stillbirth, small for gestational age and preterm birth. An independent association has been found between preterm birth and *M. genitalium* infection (Edwards *et al.*, 2006; Hitti *et al.*, 2010). A study of 1014 women showed 6.2% *M. genitalium* infection among women who gave birth or aborted but not related to pregnancy outcome (Labbe *et al*., 2002). Luki *et al*., (1998) reported one premature child with acute respiratory distress where *M. genitalium* had been transmitted from mother to the newborn.

1.3.6 Clinical appearances of *M. genitalium* infection in women

1.3.6.1 Lower genital tract infection

A significant association between *M. genitalium* and microscopic urethritis in women has been found in a study of a large number (n=7604) of Scandinavian women (Moi *et al*., 2009). A positive association of urethral inflammation with *M. genitalium* was found in three of four studies considering microscopic signs of urethral inflammation (Anagrius *et al*., 2005; Hogdahl and Kihlstrom, 2007; Moi *et al*., 2009). They had considered either >4-5 polymorphonuclear per high power field, or >10 as a sign of inflammation respectively. Some studies had different inclusion criteria, such as vaginal symptoms or discharge as non-microscopic signs as well. Significant associations were observed between *M. genitalium* and vaginal discharge (Falk *et al.*, 2005; Moi *et al*, 2009).

Among studies which found positive association between microscopic signs of urethral inflammation and *M. genitalium* only one showed significant association (Moi *et al*., 2009).Where microscopic signs were considered independent of non-microscopic signs, *M. genitalium* has been positively associated with cervical inflammation (Falk *et al.*, 2005; Moi *et al*, 2009). Studies have also found microscopic or non-microscopic signs are predictors for *M. genitalium* cervicitis (Manhart *et al.*, 2003; Falk *et al.*, 2005).

M. genitalium was reported in both industrialized and developing countries. One study in Washington (USA) detected *M. genitalium* in 7% of women attending STD clinics

who had 3.3 times greater risk of mucopurulent cervicitis (Manhart *et al*., 2003). A Swedish study showed an incidence of 50% in infected women with signs and symptoms of urethritis and/or cervicitis (Falk *et al.,* 2005). In West Africa the prevalence of *M. genitalium* among female sex workers was 26.3% and was significantly associated with cervicitis (Pepin *et al*., 2005).

1.3.6.2 Bacterial vaginosis (BV)

In one of the early reports, *M. genitalium* was found in 30% women with bacterial vaginosis (Palmar *et al*., 1991). However, Keane *et al*., (2000) did not find *M. genitalium* in any women with bacterial vaginosis (BV). Lack of association between *M. genitalium* and BV has also been studied in women with cervicitis, showing that BV is no more common in patients with *M. genitalium* (Manhart *et al*., 2003).

1.3.6.3 Pelvic inflammatory disease (PID)

Mycoplasma infection can extend to the upper genital tract and lead to PID (Fraser, 1995). Sexually transmitted microorganisms can extend to the uterine tubes by invasion through the female genital tract or via the mesosalpingeal lymphatics (Eschenbach, 1984). The main site of *M. genitalium* colonization is the genital tract which results in infection ascending from the lower to the upper genital tract due to presence of *M. genitalium* in the cervices of women with adenexitis (Uno *et al.,* 1997; Clausen *et al.,* 2001). In 1984, *M. genitalium* was first known as a cause of PID (Moller *et al.,* 1984). Laparoscopically confirmed tubal infertility has been investigated by two Danish studies where they found an association between *M. genitalium*-specific serum antibodies and laparoscopic data (Clausen *et al.,* 2001; Svenstrup *et al*., 2008). There is a significant correlation between antibodies against *M. genitalium* and *C. trachomatis* seropositivity in women with TFI (Svenstrup *et al.,* 2008). In a retrospective study a correlation was found between IgG against MgPa of *M. genitalium* and women with TFI (Clausen *et al.,* 2001). Some women with PID have shown an antibody response to *M. genitalium* (Moller *et al*., 1984).

1.3.7 Pathogenesis of *M. genitalium* infection

1.3.7.1 Epidemiology

There are relatively few epidemiological studies of *M. genitalium*, due to difficulties in culturing and diagnosing the infection. The first clinical study using PCR showed *M. genitalium* DNA (17%) in the urethral swabs of men attended a STD clinic (Jensen *et al*., 1993). *M. genitalium* in men with urethritis was found more often in the *C. trachomatis* negative NGU patients (35%) compared with chlamydial NGU (7%) indicating that *M. genitalium* and *C. trachomatis* act as separate causes of urethritis (Jensen *et al*, 1993).

The prevalence of *M. genitalium* varies among urology patients and STD patients at 8% and 29% respectively, among these who have urethritis. In asymptomatic patients prevalence rates are 0% to 9% in urologic and STD patients respectively (Jensen *et al*., 1993). Evidence has shown high prevalence rates of *M. genitalium* infection among male partners of infected women (56%) and 32% among female partners of infected men with NGU (Manhart *et al*., 2003; Cohen *et al*., 2008).

Prevalence of *M. genitalium* was 18% among women attending a STD clinic in the UK (Palmer *et al.*, 1991) while Tsunoe *et al*., (2000) found a prevalence of 12.6% in commercial sex workers in Japan as compared to 1.1% of pregnant women. Two different studies in Japan reported the prevalence of *M. genitalium* in young asymptomatic men and women as 1% and 2.8% respectively. The risk factors of infection were more lifetime sexual partners and co-infection with *C. trachomatis* (Cohen *et al*., 2008). Also the prevalence of *M. genitalium* in women from Kenya with endometritis was 16% compared with 2% in a control group (Cohen *et al*., 2002). Women with PID showed 13% positivity by PCR for *M. genitalium* in a British study compared with none in the control group (Simms *et al.*, 2003).

In the review article by McGowin and Anderson-Smits (2011) the prevalence of *M. genitalium* was 2% among women from low-risk populations (n=27,272). Also the review showed a prevalence of *M. genitalium* among high-risk patients of 0% to 42% (n=18,838 women with urogenital infections). The including criteria were specific symptom or sign, co-infection with other STDs, high-risk behaviour, study geographic

location, specificity of NAAT assay which used. Low-risk women were those not attending a STD clinic and high-risk were those attending STD clinic, or enrolled in a study for urogenital disease, or women classified as sex workers.

1.3.7.2 Immunity in *M. genitalium* infection

Specific and nonspecific immune reactions are involved between mycoplasmas and host immune system. Humoral and cell mediate immune responses are involved as described below.

1.3.7.2.1 Innate defence mechanisms

Innate immune responses in *M. genitalium* infection were observed through Toll-like receptor expression (McCowin *et al*., 2009). *M. genitalium* can active NF-κ B (Nuclear Factor-Kappa B) via TLR-2/6. Epithelial cells of genital tract are responder to STDs and express high levels TLR_2 and TLR_6 (McGowin *et al*., 2009).

1.3.7.2.2 Humoral response

During persistent infection, *M. genitalium* reactive cervicovaginal IgA and IgG antibodies were detected in women (Iverson-Cabral *et al*., 2011). They investigated antibody response in initial site of infection. Infected mice with *M. genitalium* have shown high level of antibody to MgPa that indicating persistent infection (McGowin *et al*., 2010).

1.3.7.2.3 Cell mediated immunity (CMI)

Mycoplasmas are able to induce cell to produce different types of interferon (Cole *et al*., 1985). Stimulation of host immunocytes by mycoplasmas is reflected by producing of different pro-inflammatory cytokines (Razin *et al*., 1998) such as: TNF-α, NK cells, IL-1, IL-2 and IL-6.

1.3.8 Different methods for detecting *M. genitalium*

1.3.8.1 Cell Culture

The first time *M. genitalium* was isolated (Tully *et al.*, 1981) from two men with NGU using cell culture, its growth was extremely slow and further studies, by Taylor-Robinson *et al*., (1985) and Samra *et al*., (1988) failed to culture *M. genitalium*.

However, in a Chinese study 8 strains of *M. genitalium* were isolated from urogenital specimens by culture (Luo *et al*., 1999). The strains G-37 and M-30 were chosen as the type strains of *M. genitalium* (Taylor-Robinson *et al*., 1983).

1.3.8.2 Serology tests

In epidemiological studies, the use of specific serology for diagnosis has been hampered due to cross reactions between mycoplasma species especially *M. genitalium* and *M. pneumoniae* (Lind, 1982). Jensen *et al*., (1993) used ELISA to detect IgG, IgA, and IgM from serum specimens as a part of a prevalence study. The method for antigen preparation was the same with Uldum *et al*., (1992) and no correlation could be shown between the PCR status and antibody response.

Using Triton X-114 extracted Lipid Associated Membrane Protein (LAMP) to measure *M. genitalium* antibodies (Wang *et al*., 1997) showed 38% positivity (40 of 104 patients). The results showed that 15% of LAMP positive patients were also PCR positive. The assay was validated with serum and urine specimens. Another study by Baseman (2004) followed patients using a modified method of LAMP ELISA (over 22 months follow-up): 83% of culture positive women had a positive ELISA results.

1.3.8.3 Molecular methods

The first two studies to use PCR to detect *M. genitalium* were published in 1991 (Jensen *et al*., 1991; Palmer *et al*., 1991). It was believed that MgPa DNA had an important role in pathogenesis of the infection at that time and the assays were based on a MgPa DNA sequence (Jensen *et al*., 1991). The hemi-nested PCR is an extremely sensitive assay was developed by Palmer *et al*., (1991). The studies on male urethritis, endometritis and cervicitis used a modification of a $MgPa_1$/ $MgPa_3$ assay (Totten *et al*., 2001; Cohen *et al*., 2002; Manhart *et al.,* 2003). This assay was a confirmatory test of 16S rRNA PCR (Jensen *et al*., 2003). Sasaki *et al*., (1992) described another 16S rRNA assay that was used on clinical specimens.

Real time PCR is a more recent development in *M. genitalium* diagnosis. Yoshida *et al*., (2002) published the first quantitative paper that used real time PCR with wide dynamic range PCR for detection of *M. genitalium* 16S rRNA. Specific and sensitive primer design was difficult due to cross reaction between *M. genitalium* and *M. pneumoniae*

(Yoshida *et al*., 2002). Diagnosis of *M. genitalium* based on real time PCRs is highly sensitive and specific (Yoshida *et al*., 2002).

1.4 Conclusions

This introducing chapter has reviewed the background biology, epidemiology and pathology of *C. trachomatis* and *M. genitalium*. It has shown that these are bacterial infections of both the male and female reproductive systems, with important consequences for health and fertility. Even so, our knowledge of both organisms in an infertility setting is incomplete and the work described in this thesis aims to fill some of these gaps.

As a study population, infertile couples in Iran have been chosen. That is because there are significant cultural and political sensitivities about sexually transmitted infection in Iran. Since non-marital sex is prohibited by Islam's religion, the reported prevalence of STDs in countries such as Iran could be different from other countries. However, infertility remains a significant problem with 12-15% of couples is affected in Iran. This present study intended to find the prevalence of these STDs in infertile couples in Iran by different specimens and methods. This is the first study of its kind, given that cultural sensitivity of STDs, that there are no STD clinics in Iran and no national statistics are collected. There is no study with this scale to evaluate both males and females that consider semen parameters, sperm DNA measurements, serology tests, past reproductive and medical history regards with the presence of possible infection in male and female partners of infertile couples. Therefore, the present study for first time will find prevalence and relationship of these STDs and infertility.

These studies were carried out at the Research and Clinical Centre for Infertility Yazd/ Iran which is a referral centre. Although the prevalence would vary among different population and area and culture, however, this study can reflect the overall prevalence of these STD in Iran.

2 Chapter 2: Materials and Methods

2.1 Study population and samples obtained

2.1.1 Infertile couples

Three hundred and twenty-four couples with primary or secondary infertility attending the Research and Clinical Centre for Infertility, Yazd, Iran were screened between September 2009 and October 2010. The centre is the most famous public centre of Iranian Medical Universities because of the high success rates in ART and the lowest rate of expense among other public centre in Iran. The centre location and also its reputation lead to 25-30 daily consultations by gynaecologist and urologist. People mostly come from the rural area of Yazd and other Southern provinces. Also the centre is known as a referral centre for problematic or undiagnosed cases of infertility. Therefore the centre often sees couples from all regions of Iran and even abroad.

All couples were approached with information about the study and were asked to participate unless they had: (i) abnormal karyotype; (ii) history of chemotherapy or radiotherapy treatment; (iii) previous vasectomy; (iv) low semen volume (<1.0 ml); (v) retrograde ejaculation; (vi) hypogonadotropic hypogonadism; (vii) genital tract anomaly; or (viii) female age >35 years.

Seventy four couples were excluded, leaving 250 to enter the study. Of the 250 couples enrolled (see Figure 2.1) each partner gave informed consent and completed a standardized questionnaire during an interview (see Appendix I). This included information about: age, type of infertility (primary or secondary), past history of genital tract infection in both male and female, their career, the history of using ART, tubal factor damage, endometriosis, miscarriage, having a child from the current or any previous marriage, history of varicocele and mumps in the male. In addition, all individuals provided a 2-ml blood sample (1.5-ml serum) and a 20-40 ml urine sample. Male partners also provided semen sample. The male partners were examined by a urologist and the data for any urogenital abnormality and varicocele was obtained from patient notes. The interviewer observed vaginal sonography of female partners recruited in the study and laparoscopy results are from patient notes.

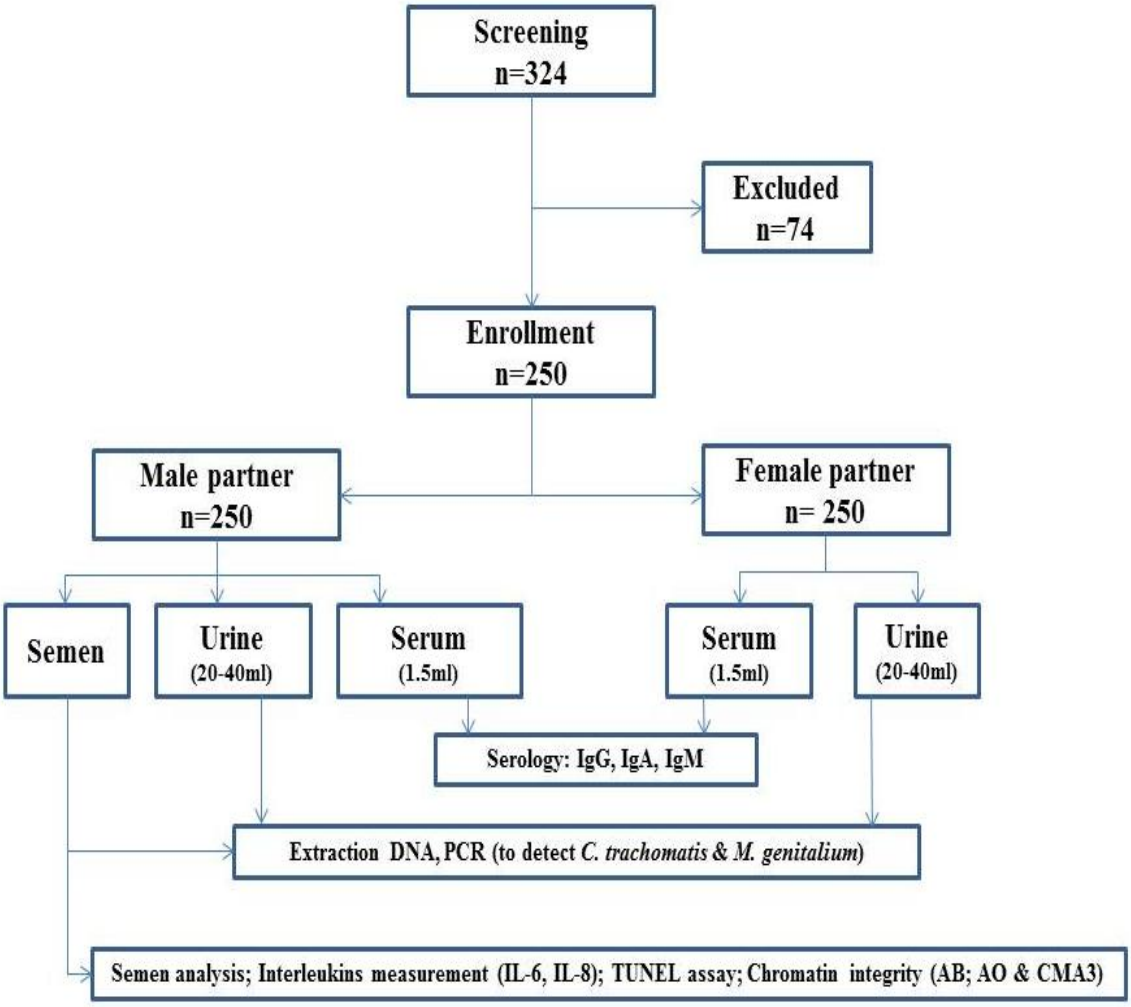


Figure 2.1: Study population and samples obtained (infertile group).

2.1.2 Control group

Two hundred and fifty pregnant women attending the antenatal clinic in the Akbary Public Health centre, Yazd, Iran were also recruited as a control group between May 2010 and September 2010. Written informed consent was obtained from all individuals and each individual provided 2 ml of blood and a 20-40 ml urine samples that were processed and stored as described in 2.2.1. Extensive attempts were also made to recruit fertile men but this was not successful.

2.1.3 Ethical approval

The study was approved by the Ministry of Health Research Ethics Committee, Iran (Declaration no=11P/88k-A, 9th Sep 2009), as well as The University of Sheffield, School of Medicine Research Ethics Committee (SMBRER147, 10th October 2010) see Appendix II.

2.2 Sample processing and transportation

2.2.1 Sample processing

Serum was separated from blood sample the same day that it was collected. Briefly, a tube without any anticoagulant was used to collect the blood and then the tube was left in a standing position for a maximum of 20-30 minutes. After that, the tube was centrifuged (blood was clotted) at 1500 g for 10 minutes then the serum removed very quickly and stored at -20ºC in the Yazd IVF centre within 6h of collection.

Semen analysis was first performed on the ejaculate on the same day (see section 2.2.2). For each patient two semen smears were prepared to assess sperm morphology and the dried slide was fixed in equal volume of Ethanol-Ether for 10 minutes, before being stored in the fridge and then stained by Papanicolaou staining method (WHO, 1999). Stained morphology slides were stored at room temperature (RT) for transportation to Sheffield and assessment by CASMA (section 2.3.6). Seminal plasma was removed after centrifuging fresh semen at 1000g 5 minutes then was stored at -80ºC before transportation back to Sheffield to measure inflammatory markers.

Two slides were also prepared for each patient to assess sperm DNA fragmentation, to achieve this the sample was first centrifuged at 1000 g for 5 minutes and the pellet resuspended in tris-buffered saline (TBS) to give an appropriate sperm concentration of

$1x10^6$. A 100µl aliquot was used for preparing the smear and slides were dried overnight. Methanol 100% was used for fixation for 1 minute; fixed slides were stored at -80ºC before transportation back to Sheffield and staining for TUNEL assay (see section 2.3.7.1).

Three semen smears were prepared to evaluate sperm chromatin assessment. The sperm concentration was adjusted again to $1x10^6$ sperm per ml, however 10µl was used to prepare the smear and air dried slides were then fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4ºC overnight for Acridine Orange (AO) and Chromomycin A3 (CMA3) staining. Air dried slides for Aniline Blue (AB) staining were fixed in 3% (v/v) buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 minutes at RT. The prepared fixed slides were stored at 4ºC in Yazd to stain and evaluate later in the study (see sections 2.3.7.2; 2.3.7.3 and 2.3.7.4). The rest of semen sample was stored at -80ºC to extract DNA (see section 2.2.3).

Urine samples for both male and female partners were stored at fridge immediately after collection and DNA extraction (see section 2.2.3) was performed within 2 days and the DNA stored at -20ºC before being transported to Sheffield for subsequent PCR (see section 2.3.2 & 2.3.3).

Semen analysis, preparing fixed slides to assess sperm morphology and sperm DNA measurements and all DNA extraction (from urine and semen) were performed in Yazd. Serology tests to detect IgA, IgG and IgM antibodies to chlamydia, PCR to detect *C. trachomatis* and *M. genitalium* (urine and semen DNA), ELISA test on seminal plasma to measure IL-6 and IL-8 levels, TUNEL assay for sperm DNA fragmentation, assessment of sperm morphology by CASMA were performed in Sheffield. Patient's follow up and sperm nuclear chromatin assessment were performed at the end of the study in Yazd (Figure 2.2).

2.2.2 Collection and examination of human semen

Men produced their ejaculates after at least 48 hours sexual abstinence and semen analysis was performed according to World Health Organisation (1999) guidelines. Briefly, after 60 min liquefaction of semen at 37ºC the microscopic and macroscopic examinations were performed. Macroscopic examination included liquefaction, appearance, volume, viscosity, pH of the semen sample. Semen pH was measured by

pH paper and semen volume was measured by reading from the base of the meniscus in a graduated test tube (BD Biosciences, Bedford, USA).

Microscopic investigation included concentration, motility, agglutination of spermatozoa and the presence of cellular elements. For the assessment of sperm concentration the haemocytometer (Hawksley, London, UK) method was used. Motility was assessed by classifying 200 spermatozoa, and grading them to progressive motility; non-progressive motility and immotility. Motility was assessed at x400 magnification on a light microscope (Carl Ziess axiostor plus, Kirchdorf, Germany).

IgA and IgG antibodies were assessed by the mixed antiglobulin reaction (MAR). The MAR tests were performed by mixing semen (5µl) with latex particles coated with human IgG or IgA (5µl). Then, a mono specific anti–human-IgG antiserum (5µl) was added to the mixture using spermMar kit (Fertipro N.V., Aalter, Belgium). The presence of IgG or IgA is shown with the formation of a mixed agglutination. The number of sperm with beads attached was counted and expressed as a percentage of the total. The diagnosis of immunological infertility is possible when 50% or more of the motile spermatozoa have adherent particles.

2.2.3 DNA extraction from urine and semen samples

DNA was extracted from first void urine obtained from infertile couples (see 2.1.1) and fertile women (see 2.1.2), as well as semen samples from infertile men (after routine semen analysis had been completed and all slides had been made) using the QIAGEN kit (GmbH, Hilden, Germany) following the manufactures instruction. All DNA was stored at -20˚C prior to performing PCR in Sheffield (see sections 2.3.2 and 2.3.3).

2.2.4 Transfer of specimens

Sera, seminal plasma, DNA and fixed slides for sperm DNA fragmentation assessment were transferred on dry ice to UK at the end of the recruitment phase of the study. Fixed slides for sperm morphology assessment (were stained in Yazd) were shipped separately at RT. Upon arrival in Sheffield the fixed slides for sperm DNA fragmentation were kept at -80˚C, whereas the rest of the samples were stored at -20˚C prior to analysis.

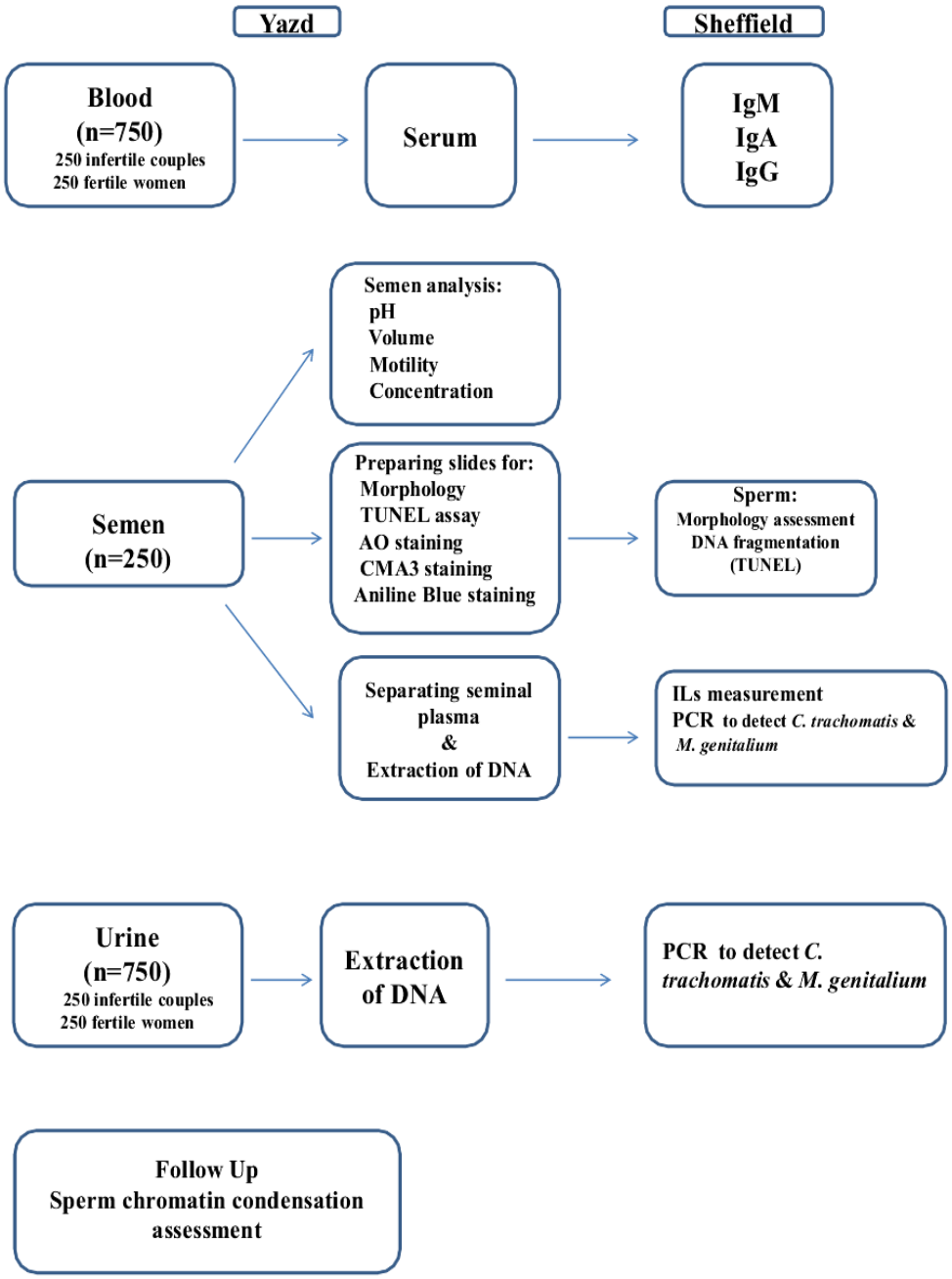


Figure 2.2: Sample processing

2.3 Analysis of stored samples

2.3.1 Immunofluorescence assay for the detection of antibodies to *C. trachomatis*

To detect specific IgG, IgA, IgM antibodies to *C. trachomatis* an immunofluorescence assay (SeroFIA^{TM} *C. trachomatis*) kit was used (Savyon, Ashdod, Israel). Briefly, purified elementary bodies (EBs) of *C. trachomatis* (supplied by the kit) were fixed onto the SeroFIA^{TM} slide wells. First serum samples were thawed, and diluted; according to the kit instructions: the dilution rate for IgG serum antibody was 1:64, IgA serum antibody titre was 1:32 and IgM serum antibody titre was 1:20. Diluted patients sera were incubated for 30 minutes at 37˚C with the antigen. Unbound serum components were removed by washing and then a fluorescein-conjugated anti-human IgG, IgA, IgM were added and incubated for 30 minutes at 37˚C. After washing to remove any unbound conjugate, slides were dried and mounted and then were examined using a Leica fluorescence microscope DMIRB (Lasertechnik Gmbh, Heidelberg, Germany). The whole well was first scanned at magnification of ×400 to observe any evidence of typical fluorescence and the well was then observed further at a magnification of ×1000 using oil immersion to confirm presence or absence of typical fluorescence in EB like morphology (small granules). Positive reactions appear as bright apple-green fluorescent EBs against a dark background (see Chapter 3, Figure 3.2). Each slide was observed by two people and a positive sample was defined when both were in agreement. Positive and negative controls were included from the kit in each slide.

2.3.2 PCR to detect *C. trachomatis* on urine and semen DNA

Two serovars were used as a positive control. Serovar E of *C. trachomatis* was isolated from a clinical source (cervical swab from Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield), and serovar LGV was provided by the University of Southampton (Southampton, UK). Confirmation of genotype was conducted by nested PCR according to method of Lan *et al* (1994). The DNA extraction QIAGEN Kit (Gmbh, Hilden, Germany) instructions were used to extract DNA from purified EBs (see sections 2.3.2 and 2.3.3) to use as a positive control in PCR. DNA extracted from EBs was checked by nested plasmid PCR and stored at -20ºC until used as a positive control.

Nested plasmid PCR was conducted according to method of Claas *et al*., (1990) and two pairs of primers were used to detect *C. trachomatis* (see Table 2.1). T_1 and T_2 were derived from sequences of the common endogenous plasmid of *C. trachomatis*, and generated a species-specific 517 bp product with all serovars of *C. trachomatis*. Primers T_3 and T_4 are internal to the primers T_1 and T_2 and amplified a 320 bp product. Primers were ordered from Eurofins MWG/Operon (Eurofins MWG/Operon, London, UK). The PCR program of 25 cycles included: denaturation 94ºC 30 seconds, annealing 42ºC 30 seconds, and extension 72ºC 30 seconds. The extension time was increased to 9 minutes (Class *et al*., 1990). Semen DNA was first tested for β-globin according to the method of Saiki *et al*., (1985) to check there were no PCR inhibitors in the samples.

2.3.2.1 PCR equipment

Micro-centrifuge tubes (Eppendorf, 0.5 & 1.5 ml), and positive displacement tips (PCR microsyringes) were purchased from Alpha Laboratories (Hampshire, UK). Laboratory pipettes were purchased from Gilson Pipetman (Villers Le Bel, France). Tech gene PCR machine with heated lid was used for all PCR applications (Teckne, Cambridge, UK).

2.3.2.2 Water

Water for PCR reactions was purified using a Milli-Ro system (Millipore Ltd, Watford Herts, UK) and this was sterilised at 15 lbs sq $inch^{-1}$ for 15 min before use.

2.3.2.3 ReddyMix™ PCR Master Mix (1.5mM $MgCl_2$)

Reddy mix (1.1 x) was purchased from Thermo scientific (ABgene, UK). The ThermoPrime *Taq* DNA polymerase; dNTPs, reaction buffer and magnesium chloride were all present in the mix. The final reaction 1X contained: ThermoPrime Taq DNA polymerase (0.625units), Tris-HCl (pH8.8 at 25ºC) (75mM), $(NH_4)_2SO_4$ (20mM), $MgCl_2$ (1.5mM), Tween 20[0.01% (v/v)], each of dATP, dCTP, dGTP and dTTP (0.2mM). It was stored at -20º C until ready for use for up to 1 year. Primary single plasmid PCR mixture was made up with Reddy Mix (43 μl), T_1 (forward) 1 μl, T_2 (reverse) 1 μl, and DNA sample 5 μl. Nested plasmid PCR reaction mixture was made up with Reddy Mix (43 μl), T_3 (forward) 1 μl, T_4 (reverse) 1 μl, and DNA sample 5 μl that was PCR product from first stage.

Table 2.1: Primers for nested PCR of *C. trachomatis* DNA

Primers	5'→ 3' Sequence
T_1 (Forward)	5'-GGA CAA ATC GTA TCT CGG-3'
T_2 (Reverse)	5'-GAA ACC AAC TCT ACG CTG-3'
T_3 (Forward)	5'-ATC CAT TGC GTA GAT CTC CG-3'
T_4 (Reverse)	5'-GCC ATG TCT ATA GCT AAA GC-3'

Table 2.2: Primers for PCR to detect *M. genitalium* MgPa (adhesin)

Primers	5'→ 3'Sequence
Mg Pa-1 (Forward)	5'-AGT TGA TGA AAC CTT AAC CCC TTGG3'
Mg Pa-3 (Reverse)	5'- CCG TTG AGG GGT TTT TCC ATT TTT GC3'

Table 2.3: Primers for PCR to detect *M. genitalium* 16S rRNA

Primers	5'→ 3' sequence
MG16-45(Forward)	5-TAC ATG CAA GTC GAT CGG AAG TAG C
MG16-447(Reverse)	5-AAA CTC CAG CCA TTG CCT GCT AG

2.3.2.4 Electrophoresis

Electrophoresis grade agarose was obtained from Melford laboratory Ltd (Ipswich, UK). A gel solution was prepared in 50 ml tris–acetate buffer (TAE) and heated in a microwave oven at 100ºC for 2 minutes. Agarose gel 0.8% (w/v) was prepared by adding 50 ml TAE buffer to 0.4 g agarose, and microwaving for 1-2 minutes. Qstep 4 DNA Ladder, and 5x DNA loading buffer were used (Yorkshire Bioscience Ltd, UK).

2.3.2.5 Tris –acetate EDTA buffer (TAE 50x)

This was made by adding to 1 litres of water the following: Tris-base (242g), Na_2EDTA (37.2g), Glacial acetic acid (57.1 mL).

2.3.2.6 Ethidium bromide stock solution (10mg/ml)

This was prepared by adding 1g of ethidium bromide to 100 ml water. The container was wrapped in aluminium foil and stored at RT. Either 5µl of this solution was put into the gel when it was being made, or after electrophoresis.

2.3.3 PCR to detect *M. genitalium* on urine and semen

PCR was looking for 16SrRNA gene (Jensen *et al*., 2003). The DNA template was supplied by the Health Protection Agency (HPA) London. The PCR is based on previous work (Jensen *et al*., 1991; 2003). The sequence of the 16S rRNA gene was determined for *M. genitalium* G_{37} (type strain) by Jensen *et al.,* 2003. From regions showing the least homology to *M. pneumoniae*, primers were chosen to amplify DNA from *M. genitalium*, and then confirmed by the MgPa primer (see Table 2.2) set (Jensen *et al*., 1991). This program included a preheating 80ºC (hot start) period and 40 cycles including, denaturation 94ºC 30-seconds, annealing 55ºC 30-seconds, and extension 72ºC 1 minute. A band of 427 bp was expected. MG16-45 and MG16-447 were used as primers, see Table 2.3. The mixture for PCR was made up with Reddy Mix (43 μl), forward primer 1 μl, reverse primer 1 μl, and DNA sample 5 μl. *M. genitalium* DNA (Health Protection Agency, London, UK) was used as a positive control.

2.3.4 Chlamydia culture

Chlamydia trachomatis was cultured and purified to use as positive control. In brief: Semi confluent cells (McCoy cells) and culture medium (EMEM+FCS+ Cycloheximide

+ antibiotic) were used to grow chlamydia before purification of EBs according to method Schachter & Wyrick (1994). After inoculation of semi confluent cells with 50µl EBs (from frozen aliquot, the concentration of which has been previously estimated to give a sufficient level of infectivity), the cells were centrifuged (Sigma, Philip Harris, UK) at 2130 g for 1 hr at 4ºC, and the cells were incubated at 37ºC and 5% CO_2 in a Sanyo (MC015-AC) incubator (Sanyo, Osaka, Japan) for 2-3 days. Infection rate was assessed by estimating the number of cells containing inclusion bodies. Infection rate above 50% was considered successful passage. For purification and storage of *C. trachomatis* EBs, sufficient flasks were needed to harvest a total of 180 ml. This was sonicated (MSE, Liverpool, UK) on ice to disrupt the cells and release EBs and centrifuged at 500 g for 10 minutes at 4ºC to deposit unwanted debris. Then the supernatant was collected and in high-speed centrifuge tubes, was centrifuged at 28,000 g for 1 hr at 4ºC to obtain a pellet of EBs. The supernatant was removed and the EB pellet was washed gently with ice-cold PBS. Then the pellet was resuspended in ice-cold Sucrose Phosphate Buffer. The EBs were aliquoted in centrifuge tubes and stored at -80ºC or in the vapour phase of liquid nitrogen.

2.3.5 *Chlamydia* purification reagents and equipment

2.3.5.1 Media

Eagle's minimal essential medium (EMEM) was purchased from Lonza (Slough, UK) and foetal calf serum (FCS) was purchased from Autogen Bioclear (Calne, UK). After the sterility checks the media were stored at 4ºC until used.

2.3.5.2 Cell lines

The McCoy cell line was used and was derived from mouse fibroblasts. It was obtained from Flow Laboratories (Rickmansworth, Herts, UK). The cell line was grown in EMEM medium. Maintenance medium for McCoy cells included EMEM and FCS.

2.3.5.3 Chlamydia culture medium

This consists of the basal medium EMEM supplemented with 10% (v/v) FCS. The following antibiotics were added to minimise the possibility of contamination: Amphotericin B (final concentration: 0.5µg/ml), Gentamicin (20µg/ml), Vancomycin (10µg/ml) and Streptomycin (200µg/ml). The other addition to growth medium was the

chemical Cycloheximide, with a final concentration of 2µg/ ml. This is an anti-mitotic agent which improves the infectivity of the *Chlamydia* in the McCoy cells. The pH of the culture media was adjusted to pH 7.4 by adding HCl.

2.3.5.4 Trypsin and Versene

This was purchased frozen in 500 ml volumes (supplied by Invitrogen Ltd, Paisley, UK). It was aliquoted into 2-4 ml volumes and stored at -20ºC until used.

2.3.5.5 Cycloheximide: [3-(2-3,5-Dimethyl-2-oxocyclohexyl glutarimide]

It was supplied by Sigma Chemical Co (Poole, UK) and dissolved in sterile purified water, to a concentration of 2µg/ml. It was stored at -20ºC.

2.3.5.6 Urografin

Urografin was obtained from Schering Health Care Ltd (West Sussex, UK) and stored at 4ºC before chlamydia purification, contains sodium and meglumine diatrizoate at concentrations of 40 mg/ml and 260 mg/ml respectively.

2.3.5.7 Sucrose Phosphate Buffer

This was made by adding to 1 litre of glass-distilled water the following: Sucrose (74.62g), Potassium di-hydrogen phosphate (0.51g), Di-potassium hydrogen phosphate (1.23g) and glutamic acid (0.72g). This was filtered to sterilise through 0.2μm pore-size membrane and then stored in aliquots at -20ºC.

2.3.5.8 Phosphate buffered saline (PBS)

PBS tablets were from Oxoid (Basingstoke, UK) and the solution prepared according to the manufacturer's instructions. The solution was sterilised. The final concentration of PBS buffer solution was 136 mM NaCl, 2.7 mM KCl, 81 mM Na_2 HPO_4, 1.47 mM KH_2P0_4, pH 7.3.

2.3.5.9 Mycoplasma contamination

Cell lines were regularly checked for the presence of *Mycoplasma spp* used the Gen-Probe Mycoplasma T.C. rapid system from Laboratory Impex System Ltd (Wimborne, Dorset, UK).

2.3.6 Assessment of sperm morphology

The stained morphology slides were assessed in Sheffield using the Hobson Sperm Tracker (Hobson Tracker Limited, Sheffield, UK). This computer-Aided Sperm Morphology Analysis (CASMA) system calculates the percentage normal forms according to WHO (1999) criteria. For each slide 200 sperm was assessed, for those slides with low sperm concentration the duplicate was assessed to count 200 sperm totally for each sample. According to WHO guidelines, a 7% sampling error occurs by counting 200 sperm (95% CI=172-228) while 10% sampling error occurs by counting 100 sperm (95% CI=80-120).

2.3.7 Sperm DNA measurements

To assess sperm DNA fragmentation and sperm nuclear chromatin condensation assessment four different techniques were used:

2.3.7.1 Detection of double strand DNA breaks (TUNEL assay)

Sperm DNA fragmentation was assessed using Calbiochem (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay) kit (Merck Chemicals Ltd, Nottingham, UK). Fixed sample was covered with 1ml TBS for 15 minutes at RT then the excess liquid was tapped off. The rehydrated fixed sample was covered with 100 µl of 20µg/ml Proteinase K, and incubated at RT for exactly 5 minutes. The slide was then washed 2-3 times with 1 ml of TBS. The rehydrated permeablised sample was covered with 100 µl 1x TdT eqilibrium buffer and was incubated at RT for 30 minutes. The permeablised equilibrated sample was then covered with 60 µl of TdT labeling reaction mix, covered with a cover slip, and place in humidified chamber at 37˚C for 60-90 minutes. After removing the cover slip, slide was twice incubated in TBS for 1 minute at RT, and the labelled sample covered with 15 µl mounting medium with propidium iodied. The slide was then covered with a cover slip and observed under FITC fluorescence at final magnification of ×1000 using oil immersion (Olympus, Tokyo, Japan). Positive and negative controls were included in each slide.

2.3.7.2 Detection of residual histones (Aniline Blue staining)

Aniline blue stains lysine-rich histones. Staining was performed using the method of Talebi *et al*., (2008) in Yazd. Briefly, air-dried smears were prepared from fresh semen

samples of each study participant (section 2.1.3), fixed in 3% (v/v) buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at RT. Each smear was stained with 5% (w/v) aqueous Aniline Blue stain in 4% (v/v) acetic acid (pH 3.5) for 5 min. Slides were assessed with light microscopy (Olympus, Tokyo, Japan), at final magnification ×1000 using oil immersion. Unstained or pale blue stained were considered as normal spermatozoa whereas dark blue stained were classified as abnormal spermatozoa. A total of 200 spermatozoa were counted in each slide. Positive and negative controls were included in each slide.

2.3.7.3 Evaluation of protamination (Chromomycin A3 staining)

Staining was performed using the method of Talebi *et al*., (2008) in Yazd. Smears were first dried and then fixed in Carnoys solution (methanol/glacial acetic acid, 3:1) at 4ºC overnight. Each slide was treated with 100 µl of CMA3 (Sigma, St Louis, MO, USA) (0.25 mg/ml in McIIvain buffer consists of 7 ml citric acid, 0.1 M + 32.9 ml Na_2HPO_4,$7H_2O$, 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 20 min. After staining, the slides were washed in McIIvain buffer and mounted with buffered glycerol (1:1). A total of 200 spermatozoa were counted in each slide. Bright yellow stained were considered abnormal (Chromomycin-reacted spermatozoa-CMA_3+) and yellowish green stained were classified normal (non-reacted spermatozoa-CMA_3−). The slides were viewed under fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter at final magnification of ×400 using oil immersion. Positive and negative controls were included in each slide.

2.3.7.4 Detection of sperm chromatin condensation anomalies (Acridine Orange staining)

Sperm DNA integrity was determined by Acridine Orange (AO) staining using the method Talebi *et al*., (2008) in Yazd. The smears were first air-dried and then fixed overnight in Carnoy's solution. Each sample was stained for 10 min in freshly prepared AO (Sigma Chemical Co, St Louis, USA) 0.19 mg/ml in McIIvain phosphate– citrate buffer (pH 4) for 10 min. Smears were assessed on the same day with the aid of fluorescent microscope (Zeiss Co., Jena, Germany) with a 460-nm filter at final magnification of ×400 using oil immersion. The duration of illumination was limited to 40s per field because the fluorescence faded after this time. A total of 200 spermatozoa were counted in each slide. The percentage of green (normal double-stranded DNA) and

orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated. Positive and negative controls were included in each slide.

2.3.8 Assessment of seminal interleukins

2.3.8.1 Human Interleukin-6

Seminal plasma was first thawed and then was diluted with PBS (1:2) before testing. Each diluted sample (100 mL) was tested with a commercial quantitative sandwich enzyme immunoassay (R & D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. A monoclonal antibody specific to IL-6 had been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any IL-6 present was bound by the immobilized antibody. After washing to remove any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. After a further washing step to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells for 30 minutes and the color was developed in proportion to the amount of IL-6 bound in the initial step. Color development was stopped by adding 50 mL of stop solution to each well, and the intensity of color was measured. The optical density of each well was determined within 30 minutes, using a microplate reader (Dynex technologies, Chantilly, USA) set to 450 nm. The average reading for each standard, control and sample was subtracted of zero standard optical density. A standard curve was constructed for each 96-well plate by plotting the mean absorbance for each standard on the y axis against the concentration on the x axis: a best fit curve was drawn through the points on the graph. The minimum detectable dose of IL-6 was less than 0.7pg/ml.

2.3.8.2 Human Interleukin-8

Seminal plasma was first thawed and then was diluted with PBS (1:4) before testing. Each diluted sample (100 mL) was tested with the use of a commercial quantitative sandwich enzyme immunoassay (R & D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. Essentially, the assay method for IL-8 was as described above for IL-6. The minimum detectable dose (MDD) was 1.5- 7.5 pg/mL the mean MDD was 3.5 pg/ml.

2.4 Follow Up

Patients were followed up 24 months after their enrollment into the study. Briefly this involved collecting from their medical records what kind of treatment and diagnostic procedure were performed including the result of: vaginal sonography, laparoscopy, hysterosalpingpgraphy, which type of ART was undertaken (if applicable). The final result of pregnancy outcome (live birth; miscarriage; ectopic pregnancy and still birth) mode of delivery (natural or caesarean) and sex and weight of baby were obtained by telephone interview.

2.5 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. During the follow up all of clinical data were recorded in a spreadsheet then entered into SPSS. The power of the study was 80% with a P-value ≤ 0.05 used as the level of significance. See Appendix III for calculation (it was calculated according to literature based on reported *C. trachomatis* infection prevalence in Iran).

The data were first printed out and after comparing with raw data and confirming it was accurate, analysis was performed. Chi-square test was employed to compare positivity of *C. trachomatis* by different tests between couples (Chapter 3). Distribution tests were performed in the SPSS package as a routine pre requisite to testing by the t-test (Chapter 4). Descriptive statistic and an independent sample T test were used for data with normal distribution. Mann-Whitney U test was used for non- parametric data (Chapter 4 & 5). Spearman rank correlation test was used to evaluate the relationship between the levels of IL-6 and IL-8 with semen parameters (Chapter 4). To investigate the correlation between variables Pearson correlation test was used (Chapter 5). Odds ratio and 95% Confidence Interval (95% CI) were used to investigate relationships between past medical and reproductive history and infection (Chapter 6). Logistic regression was used to examine confounding factors among clinical data obtained in the study population (Chapter 6). A P-value of ≤ 0.05 was considered as indicating a significant difference.

3 Chapter 3: Prevalence of *C. trachomatis* & *M. genitalium* in infertile couples & fertile women

3.1 Introduction

The prevalence of STDs including *C. trachomatis* and *M. genitalium* depends on sex, age, sexual activity, study population and the diagnostic methods used (Casin *et al*., 2002; Dorey *et al*., 2012). Therefore their incidence in many different papers published is highly variable. Many studies have investigated the prevalence of *C. trachomatis* in subfertile women and/or men by different methods, specimens and population (see Appendix IV).

Researchers in Brazil found the prevalence of *C. trachomatis* to be 52.8% among infertile women using PCR of endocervical samples (de Lima Freitas *et al.,* 2011). The prevalence of this infection in asymptomatic subfertile women in Germany was 1.0% by PCR on urine sample (Eggert-Kruse *et al*., 2003).

In Nigeria study on student and non-student population showed overall prevalence of *C. trachomatis* 29.4% using ELISA. However, multi partnership was seen more in student (71.2%), compared with non-student (28.8%) populations (Ikeme *et al*., 2011). When Ligase Chain Reaction (LCR) was used on semen from men in infertile couples Eggert-Kruse *et al*., (1997) found no positive results from semen samples, whereas Bollmann *et al*., (2001) found 4.0% positivity by PCR. In a study by Eggert-Kruse *et al*., (2003) a large number of asymptomatic subfertile men (n=707) were investigated for *C. trachomatis* using urine samples and the prevalence of this infection was 1.8% by PCR (Eggert-Kruse *et al*., 2003).

Seroprevalence study of women of reproductive age in Bangladesh indicates high prevalence of *C. trachomatis* infection among both symptomatic (21.6%) and asymptomatic (44.1%) women (Mahmud *et al*., 2011). Their data shows specific *C. trachomatis* IgG antibody in pregnant (24.1%) and non-pregnant (4.6%) women that indicates high prevalence of chlamydial infection in pregnant women in Bangladesh (Mahmud *et al*., 2011). Elevated titre of IgG anti-chlamydia is considered as a sign of previous infection and was found in 20.8% of subfertile women and 12.6% of their partners moreover it was more frequent in patients with seropositive partner (Eggert-Kruse *et al*., 1997).

C. trachomatis as an important bacterial cause of infertility is diagnosed by testing urine sample in men (Eley and Pacey, 2011) and among 11 studies, 56% of subjects showed

C. trachomatis positivity in urine and semen samples, 23% in semen, and 20% in urine in infertile men (Eley and Pacey, 2011). Two studies by Idahl and colleagues (Idahl *et al*., 2004; 2007) investigated prevalence of IgG, IgA, IgM antibodies to *C. trachomatis* and also DNA positivity in infertile couples. They showed in 2004 that the IgG prevalence was 24.2% and 20.1% in infertile women and men respectively. Urine DNA positivity was 6.8% and 7.1% in infertile women and men respectively.

The lowest reported male infertility was a cohort study in Canada (Domes *et al*., 2012) which showed 0.3% positivity when urine and semen samples were tested by strand-displacement amplification (SDA) assay. Since the criterion for screening should be considered based on the prevalence of *C. trachomatis* in studied populations (Domes *et al*., 2012), screening is not recommended in such a low prevalence population.

Studies from Iran showed *C. trachomatis* prevalence was 12.6% among women attending obstetrics and gynaecology clinics in Tehran/Iran using PCR (Chamani-Tabriz *et al*., 2007) whereas Fatholahzadeh *et al*., (2012) showed 20.76% and 9.23% in symptomatic and asymptomatic women respectively (Fatholahzadeh *et al*., 2012). A prevalence of *C. trachomatis* of 9.3% in Iranian males with urethritis, was reported using cell culture (Ghanaat *et al*., 2008) and prevalence of *C. trachomatis* in women with cervicitis was 11.6% using serum antibody measurement (Bakhtiari *et al*., 2007). A new study has revealed the 20% *C. trachomatis* in symptomatic men using PCR on urine samples and 4% in asymptomatic men (Yeganeh *et al*., 2013) .A recent study in North East of Iran on male patients with urethritis observed 10.6% positivity by PCR (Ghazvini *et al*., 2012) and in the same province the incidence of *C. trachomatis* and *M. genitalium* in pregnant women was 13.7% and 1.02% respectively (Haghighi-Hasanabad *et al*., 2011). Regional differences are considered later (3.3.1).

In contrast to *C. trachomatis*, *M. genitalium* is not well studied in infertile couples and its prevalence as an STD is also highly variable. Although the prevalence among male partners of infertile couples was reported 18.3% in Tunisia (Gdoura *et al*., 2008), the prevalence among patients from STD clinic or genito-urinary (GU) clinic is different (McGowin and Anderson-Smits, 2011): 7.3% positivity among women attending STD clinic and less than 5% among women attending GU clinic who were considered low risk patients (McGowin and Anderson-Smits, 2011).

Study from Iran showed *M. genitalium* prevalence in asymptomatic men (2%) and symptomatic (12%) men (Yeganeh *et al*., 2013) and The prevalence of *M. genitalium* in Iranian men with NGU was reported to be 0.8% (Salari *et al*., 2003) and in Iranian women with genital tract complain was reported 3.3% (Mirnejad *et al*., 2011).

Given this heterogeneity, the aim of this first chapter is to investigate the incidence of *C. trachomatis* and *M. genitalium* infection in the 250 infertile couples recruited to this study as well as the 250 pregnant women recruited as controls. This was undertaken using different methods (NPPCR, serology) on three clinical samples (blood, urine and semen). The serology tests (IgA & IgM) indicates the acute or current infection. PCR shows current or persistent infection and IgG shows past exposure or infection. The demographic characteristics of the study participants are shown and the prevalence data compared to controls. The concordance within couples is also examined.

3.2 Materials and Methods

To examine the prevalence of both organisms in the study population and control group, NPPCR to detect *C. trachomatis* was undertaken as described in section 2.3.2 and PCR to detect *M. genitalium* undertaken as described in section 2.3.3. PCR was performed on all urine samples and on the semen samples from male partners of infertile couples. In addition, serological examination of IgG, IgM and IgA antibodies to *C. trachomatis* was performed as described in 2.3.1 on sera obtained from both partners of infertile couples and on sera from fertile women. Basic demographic data was obtained from the questionnaire administered on enrollment (see section 2.1.1).

3.3 Results

3.3.1 Demographic characteristics of study participants

Table 3.1 shows the demographic characteristics of the 250 infertile men and women and the 250 fertile women that participated in the study. Their ages ranged from 15 to 52 years of age, with the median age of infertile males being 32 (range 21-52), the median age of infertile women being 28 (range 15-35) and the median age of the fertile women in the control group being 28 (range 16-39). Most of the infertile (70.4%) or fertile (78.4%) women were aged between 20-30 years, whereas most of infertile men

(71.2%) were over 30 years old. The table shows primary infertility was seen in 182 couples (72.8%) and secondary infertility was seen in 68 infertile couples (27.2%). The duration of infertility was 5.78 ± 3.48 and 6.27 ± 3.58 years among couples who had primary and secondary infertility respectively. Among infertile couples 1.2% of men and 16.0% of women were unemployed in comparison to 22.0% of fertile women. A total of 140 (56.0%) of the men were non-manual worker and 107 (42.8%) were manual worker. In contrast, 60 (28.6%) of the infertile women were non-manual worker and 150 (71.4%) of them were manual worker. Therefore the couples considered mostly were from a working class population. Among infertile couples 1.2% of men and 16.0% of women were unemployed in comparison to 22.0% of fertile women. A total of 49 (19.6%) of the men were smokers and 32 (12.9%) were drivers. In contrast, none of the infertile women were smokers or drivers.

3.3.2 Prevalence of *C. trachomatis* in the study population

To determine the prevalence of *C. trachomatis* among the study population NAAT and serology tests were performed on DNA (from FVU and semen samples) and serum of infertile couples and fertile women. Detection of chlamydia using PCR on urine DNA showed 4.4% positivity in the male partners of infertile couples (Table 3.2). The extracted DNA from semen was first tested for β-globin according to the method Saiki *et al* (1985) to check there were no PCR inhibitors in the samples. All of semen DNA was negative for *C. trachomatis* testing by PCR. The prevalence of *C. trachomatis* in female partners of infertile couples by PCR on urine samples showed 4.8% positivity (Table 3.3). Figure 3.1 shows an example of a 1% (w/v) agarose gel with NPPCR that includes a positive and negative control as well as positive and negative samples. The expected band for the positive sample was shown at 320 bp which is the same size as positive control.

The serology test for detection of IgA, IgM and IgG antibodies to *C. trachomatis* showed 0.0%, 1.2% and 18.0% respectively positivity (Table 3.2) in sera of male partners of infertile couples. Figure 3.2 shows the typical immunofluorescence observed in positive and negative samples. The serology test for detection of IgA, IgM and IgG antibodies to *C. trachomatis* showed 0.0%, 4.0% and 15.6% respectively positivity in sera of female partners (Table 3.3).

C. trachomatis past exposure, IgG positive, was seen more frequently in the >30 years age group for men (Table 3.2) and women (Table 3.3), PCR positivity was seen more commonly for both men and women in age group of 20-30 years. Three out of forty five IgG positive men were detected positive by PCR (6.6%) and one out of thirty nine IgG *C. trachomatis* past exposure, IgG positive, was seen more frequently in the >30 years age group for men (Table 3.2) and women (Table 3.3), PCR positivity was seen more positive women were detected positive by PCR (2.5%).

3.3.3 Prevalence of *C. trachomatis* in fertile women (control group)

Using PCR on urine DNA from fertile women (n=250) did not find evidence of *C. trachomatis*. The prevalence of *C. trachomatis* using serology test in fertile women showed 12.8% positivity for IgG. All serum samples were tested for IgM and IgA as well but no positive samples were found (Table 3.4).

3.3.4 Prevalence of *M. genitalium* in the study population and control group

Urine samples of all participants (infertile couples & fertile women) and semen DNA was tested by PCR to detect 16S rRNA gene of *M. genitalium*, but no positive samples were observed. Figure 3.3 shows a 0.8% (w/v) agarose gel which is testing MgPa and 16S rRNA gene of *M. genitalium* DNA with their band size as was expected (see section 2.2.3) and was used as positive control.

Table 3.1: Demographic characteristic of the study participants

Variable		Infertile group		Fertile female (n=250)
		Male (n=250)	Female (n=250)	
Age: Median (range)		**32 (21-52)**	**28 (15-35)**	**28 (16-39)**
Age: N (%)	**<20**	**0.0 (0.0)**	**6 (2.4)**	**18 (7.2)**
	20-30	**72 (28.8)**	**176 (70.4)**	**196 (78.4)**
	>30	**178 (71.2)**	**68 (27.2)**	**36 (14.4)**
Infertility: N (%)	**Primary**	**182 (72.8)**		**n/a**
	Secondary	**68 (27.2)**		
Infertility duration:(Mean±SD)	**Primary**	**5.78±3.48**		**n/a**
	Secondary	**6.27±3.58**		
Employment: N (%)	**Employed**	**247 (98.8)**	**210 (84.0)**	**195 (78.0)**
	Unemployed	**3 (1.2)**	**40 (16.0)**	**55 (22.0)**
Employment: N (%)	***Manual**	**107 (42.8)**	**150 (71.4)**	**n/a**
	†Non-Manual	**140 (56.0)**	**60 (28.2)**	
Driver: N (%)		**32 (12.9)**	**0.0 (0.0)**	**n/a**
Smoker: N (%)		**49 (19.6)**	**0.0 (0.0)**	**n/a**

***Manual work refers to working class population.**

†Non-manual work refers to middle class population.

Table 3.2: Prevalence of *C. trachomatis* infection in male partners.

Age (years)	No.	Number of *C. trachomatis* positive samples (%)			
		Serology IgA	Serology IgM	PCR	Serology IgG
< 20	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
20-30	72	0 (0.0)	2 (2.7)	6 (8.3)	11 (15.3)
>30	178	0 (0.0)	1 (0.4)	5 (2.8)	34 (19.1)
Total	250	0 (0.0)	3 (1.2)	11 (4.4)	45 (18.0)

Table 3.3: Prevalence of *C. trachomatis* infection in female partners.

Age (years)	No.	Number of *C. trachomatis* positive samples (%)			
		Serology IgA	Serology IgM	PCR	Serology I gG
<20	6	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
20-30	176	0 (0.0)	7 (3.9)	9 (5.1)	24 (13.6)
>30	68	0 (0.0)	3 (4.4)	3 (4.4)	15 (22.0)
Total	250	0 (0.0)	10 (4.0)	12 (4.8)	39 (15.6)

Table 3.4: Prevalence of *C. trachomatis* infection in fertile women (control).

Age (years)	No.	Number of *C. trachomatis* positive samples (%)			
		Serology IgA	Serology IgM	PCR	Serology I gG
<20	18	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
20-30	196	0 (0.0)	0 (0.0)	0 (0.0)	20 (10.2)
>30	36	0 (0.0)	0 (0.0)	0 (0.0)	12 (33.3)
Total	250	0 (0.0)	0 (0.0)	0 (0.0)	32 (12.8)

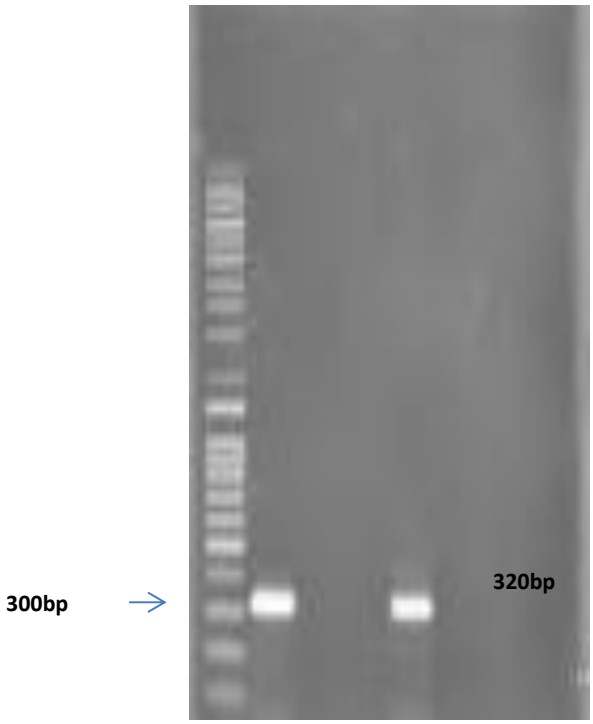
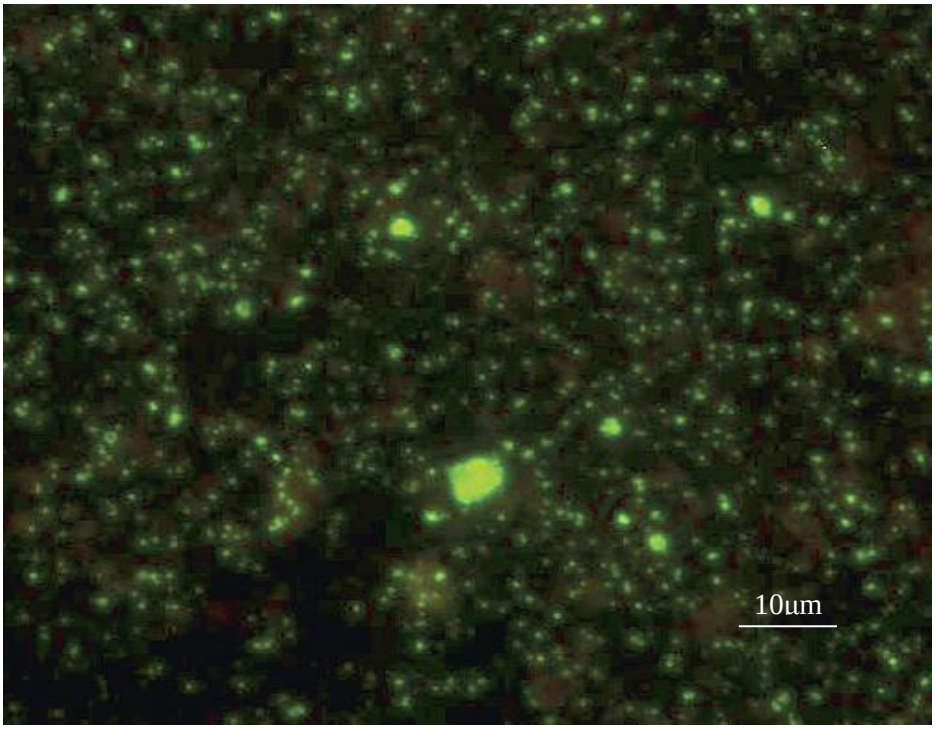
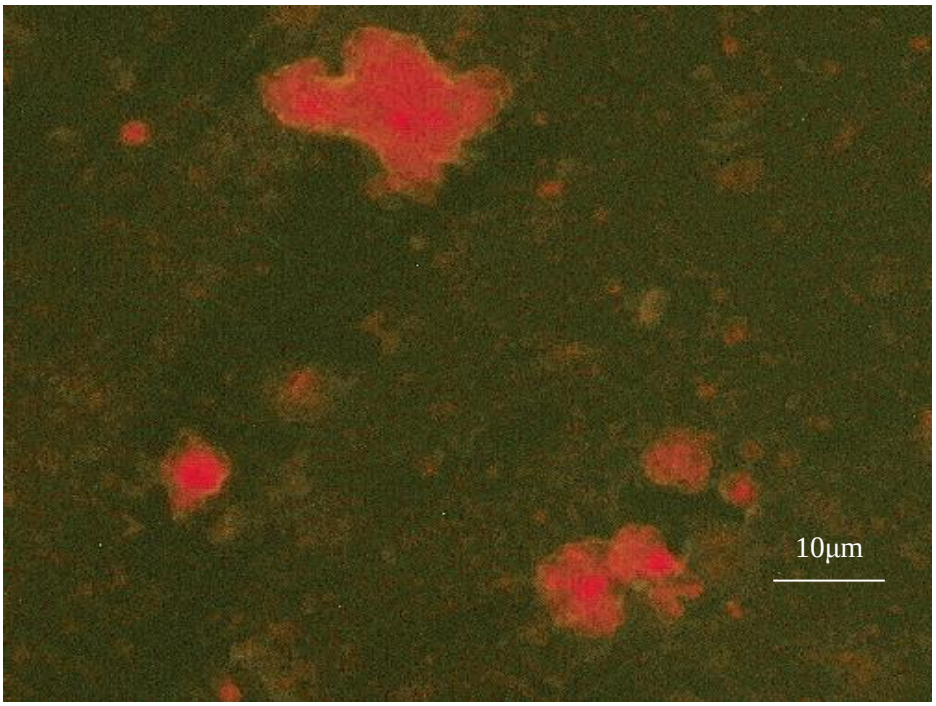


Figure 3.1: A 1.0% (w/v) agarose gel showing nested plasmid PCR on FVU samples (urine DNA) to detect *C. trachomatis*. Lane 1: DNA Ladder; Lane 2: Positive control; Lane 3: Negative control; Lane 4: Negative sample; Lane 5: Positive sample.



A



B

Figure 3.2: MIF staining using serum samples to show antibody to *C. trachomatis* (IgA, IgG, and IgM). A: showing positive reactions appear as bright apple-green fluorescent EBs against a dark background. B: negative reaction.

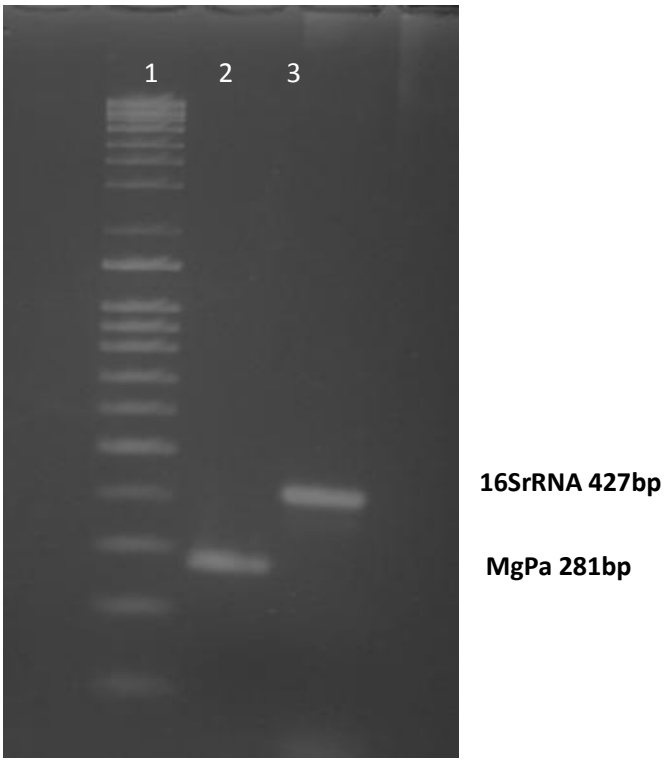


Figure 3.3: A 0.8% (w/v) agarose gel showing PCR on *M. genitalium* DNA. Lane 1: DNA Ladder; Lane 2: Positive control MgPa gene; Lane 3: Positive control 16SrRNA gene.

3.3.5 Concordance of *C. trachomatis* infections within couples

To examine concordance of *C. trachomatis* infections within infertile partnerships, the incidence of male and female IgM positive results (Table 3.5), DNA positive results (Table 3.6) and IgG positive results (Table 3.7) were examined by Chi-square analysis. Briefly, among IgM positive female partners (n=10) no positivity was seen in their partners (Table 3.5). In only one couple, both partners had positive urine DNA (Table 3.6), and from 45 men with IgG positive for *C. trachomatis*, only 9 of their partners were positive as well (Table 3.7). There was no statistical difference between the incidences of infection among couples using these three diagnostic tests, and clearly within partnership concordance was not always seen.

Although numerical differences did exist, for example, forty five men had chlamydia IgG and three of the corresponding 45 specimens (1.2%) were positive by PCR on urine DNA. Thirty nine women had chlamydia IgG and one of the corresponding 39 specimens (0.4%) was positive by PCR urine DNA. Antibody IgM to chlamydia was observed in two of forty five men and seven of thirty nine women who had chlamydia IgG.

Table 3.5: Concordance of IgM antibody to *C. trachomatis* in infertile couples.

	IgM+(♂)	IgM−(♂)	Total	Pearson chi-square Value(df)	P-value
IgM+(♀)	0*	10	10	1.27(1)	0.884
IgM−(♀)	3	237	240		
Total	3	247	250		

Table 3.6: Concordance of *C. trachomatis* urine DNA in infertile couples.

	DNA+(♂)	DNA−(♂)	Total	Pearson chi-square Value(df)	P-value
DNA+(♀)	1*	11	12	0.358(1)	0.450
DNA−(♀)	10	237	238		
Total	11	239	250		

Table 3.7: Concordance of IgG antibody to *C. trachomatis* in infertile couples.

	IgG+(♂)	IgG−(♂)	Total	Pearson chi-square Value(df)	P-value
IgG+(♀)	9	30	39	0.807(1)	0.245
IgG−(♀)	36	175	211		
Total	45	205	250		

*** Chi-square tests requires a minimum cell size of 5 observation, therefore the test result must be regarded with some caution.**

3.4 Discussion

The study was carried out to determine the prevalence of *C. trachomatis* and/or *M. genitalium* among infertile couples in Yazd and is the first large scale study undertaken using NAAT and serology in Iran. The main results of this study show that the incidence of *C. trachomatis* is comparable with other similar studies, but there was no evidence of *M. genitalium* and there is a low level of concordance within couples.

To assess the prevalence of both micro-organisms, a non-invasive technique was carried out using FVU samples. The prevalence of *C. trachomatis* following NPPCR testing on these samples (urine DNA) showed 4.4% of the male partners of infertile couples and 4.8% of the female partners of infertile couples were infected. Also serum samples were obtained from infertile couples to detect IgA, IgM and IgG antibodies to *C. trachomatis* and this showed that the IgM prevalence was 1.2% and 4.0% in the male and female partners of infertile couples respectively. The IgG prevalence was 18.0% and 15.6% in the male and female partners of infertile couples respectively. The IgA was not observed in the serum samples and this might be due to the half-life of IgA antibodies to *C. trachomatis* which is usually less than one week and normally used as an indicator of acute infection (Cevenini *et al*., 1984; Fresse *et al*., 2010).

The semen samples (semen DNA) were tested for *C. trachomatis* by NPPCR, and no positive samples were detected. This is similar to a report that showed a higher number of positive results are found in urine samples (Hamdad-Daoudi *et al*., 2004). Conversely, Gdoura *et al*., (2008) found more positivity in semen samples of infertile men from Tunisia, and this is similar to previous work carried out in Sheffield (Kokab *et al*., 2010). In a review article by Eley and Pacey (2011), 11 studies were reviewed and C. *trachomatis* could be detected 56% of the time in semen and urine, but only 20% in urine and 23% in semen. To date the preferred test specimens is FVU for men and vulvovaginal swab for female (Wiggins *et al*., 2009). An alternative specimen from men in the infertility work up is semen (Wisnieswki *et al*., 2008; Eley and Pacey, 2011) and this could provide more information about upper genital infection but there is no currently approved test for *C. trachomatis* detection in semen specimen (Eley and Pacey, 2011).

Given the presence of PCR inhibitors in FVU and semen samples including urea in urine and polyamines in semen (Eley and Pacey, 2011) there are other explanations for the low *C. trachomatis* detection in this study. First, the kit used for DNA extraction was not specific for urine or semen sample but was for general body fluid DNA extraction. This might be the main weakness of the study. Furthermore, spectrophotometry to measure DNA concentration was not performed in Yazd because of was not available, however random DNA concentration was measured for some urine samples (n=50) in Sheffield. In addition all of semen DNA were checked by β-globin PCR (n=250). Second, quantitative polymerase chain reaction (qPCR) was not used, and so the negative specimens (urine or semen) could have contained very low copy numbers of *C. trachomatis* DNA (or *M. genitalium*) that were not detected in the semi-quantitative PCR method used. Third, another possible reason is that some DNA might suffer degradation (freeze and thaw) following direct sample storage and shipment from Iran. Unfortunately, there was clearance delay in the airport in the UK for 36 hours. Fourth, volume of specimens (FVU) is a factor that could impact on the sensitivity of NAATs, however, Moncada *et al*., (2003) have stated that PCR performed equally well at all volumes (Moncada *et al*., 2003). The conclusion is that the low positivity might be a genuine finding or due to a non-specific DNA extraction kit. Positive results were also confirmed by sequencing.

Both urine and semen DNA were examined by PCR to detect *M. genitalium* but no positive samples were found. The negative results for *M. genitalium* might be because: (i) *M. genitalium* does not have cell wall and inducing osmotic lysis following washing step during DNA extraction lead to negative result (Jensen, 2006), (ii) very low prevalence of infection (iii) the inappropriate kit for DNA extraction from urine and semen (discussed above) (iv) the PCR method used (v) the economic situation of patients (they could afford to pay for their treatment were recruited into study).

The prevalence of *M. genitalium* in Iranian men with NGU was reported to be 0.8% (Salari *et al*., 2003) and in Iranian women with genital tract complain it was reported to be 3.3% (Mirnejad *et al*., 2011). Data from a recent study (Yeganeh *et al*., 2013) in Iran showed 12% and 2% positivity for *M. genitalium* in symptomatic and asymptomatic men respectively. Also they found *M. genitalium* infection significantly associated with low educational level and therefore low economic situation. Therefore our results might

be caused of selection bias as poorer people who cannot afford treatment in Iran (as people have to pay in a public medical centre) are not recruited then it can affect the prevalence of this organism in present study. Therefore, because of the asymptomatic study population and the low prevalence of this organism, the conclusion is that the low positivity might be genuine finding. This is supported by the fact that the PCR worked properly (see Figure 3.3).

In fertile women as control group only chlamydia IgG was observed (12.8%) with no positive urine DNA for *C. trachomatis* or *M. genitalium*. As expected the fertile women had lower positive serology, however it was not statistically significant (OR=1.26; 95%CI= 0.75-2.08). Positive IgG serology is suggesting prior exposure to *C. trachomatis*, and because IgA and IgM were negative suggests that infection was not in its acute phase. This would also explain why urine DNA was negative for *C. trachomatis* and *M. genitalium*. In these cases it might be possible that their immunologic response has cleared the infection or they had successful treatment.

There was no concordance within couples with regard to *C. trachomatis* infection positivity. This is in contrast with other studies that found high rate of concordance within couples (Quinn *et al*., 1996; Keane *et al*., 2000; Idahl *et al*., 2004, Guerra-Infante *et al*., 2005). The most likely reason for this is the low number of patients with positive serology or PCR tests and also the type of specimen which used to detect *C. trachomatis*. Previous studies which have found concordance have used urethral swab, and culture as their diagnostic method. Also it is not a surprising finding, since spermatozoa play a vehicle role for *C. trachomatis* to transport to female genital tract (Suarez & Pacey, 2006) and in this study sperm were not infected.

Other Middle Eastern countries like United Arab Emirates studied *C. trachomatis* prevalence in women that found 2.6% in endocervical swab specimens by rapid immunoassay (Ghazal-Aswad *et al*., 2004). In Jordan, the prevalence of genital chlamydia infection in symptomatic and asymptomatic patients (male & female) was 4.6% in patients with urethritis or cervicitis but no positives were observed in asymptomatic patients (Awwad *et al*., 2003). In infertile women in Egypt, the prevalence of chlamydia IgG and IgM antibodies in serum were 8.0% and 2.7% (Abd-Raboh *et al*., 2011). The results of serology test in the present study is similar to some

European studies (Idahl *et al*, 2004; Hamdad-Daoudi *et al*., 2004) and lower than rates reported from Germany (Eggert-Kruse *et al*., 2001).

With regard to the *M. genitalium* prevalence in infertile couples, the incidence and role of this organism has been less well studied in comparison to *C. trachomatis* and its role on human infertility is still not clear. In infertile men 5% positivity was shown by PCR on semen sample and that was a very high concordance between detection of mycoplasmas DNA in semen and first void urine (Gdoura *et al*., 2007). Among asymptomatic women and men aged 21-23 the prevalence was reported 2.3% and 1.1% respectively (Anderson *et al*., 2006). *M. genitalium* has been implicated in infertility by compromising the function of the uterine tubes, and the role of *M. genitalium* in non-gonococcal PID (Clausen *et al*., 2001; Svenstrup *et al*., 2008). Significant relationship between *M. genitalium* and tubal infertility (confirmed by laparoscope), have been observed in two Danish studies (Clausen *et al*., 2001; Svenstrup *et al*., 2008). Infertile women were more likely than fertile women to have *M. genitalium* infection in cervical swabs using PCR (Grzesko *et al*., 2009) that predict upper tract infection. As NAAT studies show association between current infection and infertility, serological studies can be useful in determining recent or long term infections.

In conclusion, non-invasive screening for *C. trachomatis* and *M. genitalium* in infertile couples was performed and illustrates the incidence of these infections are comparable with other studies. The present study found no *M. genitalium* positivity and that might be caused of the fact that treatment is not free in Iran and people recruited in this study from a potentially biased sample who are economically better off.

After defining the *C. trachomatis* and/or *M. genitalium* prevalence in population, this study goes on to look at the relationship of this infection and semen parameters and semen inflammatory markers. This is in order to see how past and current infections might affect male fertility.

4 Chapter 4: Correlation between chlamydial infection, inflammatory markers and semen quality

4.1 Introduction

There is controversy whether semen quality may be compromised by genitourinary infection in men (Gonzales *et al*., 2004). Studies show that male accessory gland infection (MAGI) may negatively interfere with semen parameters. During MAGI many inflammatory markers are released in the high levels that lead to the negative effect on semen parameters (Rowe *et al*., 1993; Agarwal *et al*., 2003, La Vignera *et al*., 2011). White blood cells (WBC) are attracted to the site of infection because of existence of pathogens and tissue damage. Macrophages produce inflammatory cytokines in response to chronic inflammation, foreign antigens and pathogens (Barratt *et al*., 1990). Cytokines have essential functions in the mediation of inflammatory responses and in the reproductive physiology of women and men (Henderson *et al*., 1996). Cytokines are soluble mediators and regulatory protein produced by leucocytes and other cells (lymphoid and non-lymphoid cells) that play a key role in both innate and acquired immune responses that control inflammation.

The enzyme peroxidase that is inside the phagosomes of polymorphonuclear granulocytes (PMN) can make reactive oxygen species from hydrogen peroxide (H_2O_2) hypoclorous acid (HCl), hydroxyl (OH) and superoxide radicals (O_2^-). The aim of these oxygen species are to destroy pathogens and this normally takes place inside of phagolysosome, but these can leak out of the PMN and damage surrounding tissue. These reactive oxygen species (ROS) are neutralized by antioxidants that normally exist in the seminal plasma and in the genital tract secretion (Comhaire *et al*., 1999; Gonzales *et al*., 2004; Tremellen & Tunc, 2010). In normal condition there is a balance between generation of ROS and antioxidants. Increasing ROS generation is what happens during WBC activation or infiltration that cannot be neutralized by the antioxidant capacity of genital fluid. The reason might be accessory sex gland dysfunction because of infection. The excessive ROS damage sperm membrane and sperm DNA (Aitken *et al*., 2003; Tremellen & Tunc, 2010). Moreover, tissue damage during infection stimulates an inflammatory reaction and the generation of interleukin 1 (IL-1). The effect of IL-1 on PMN leads to IL-8 and ROS secretion. Also IL-1 stimulates macrophages, which are a source of IL-8 and IL-6. Stimulated macrophage also produces hepatocyte growth factor (HGF) see Figure 4.1 (Comhaire *et al*., 1999).

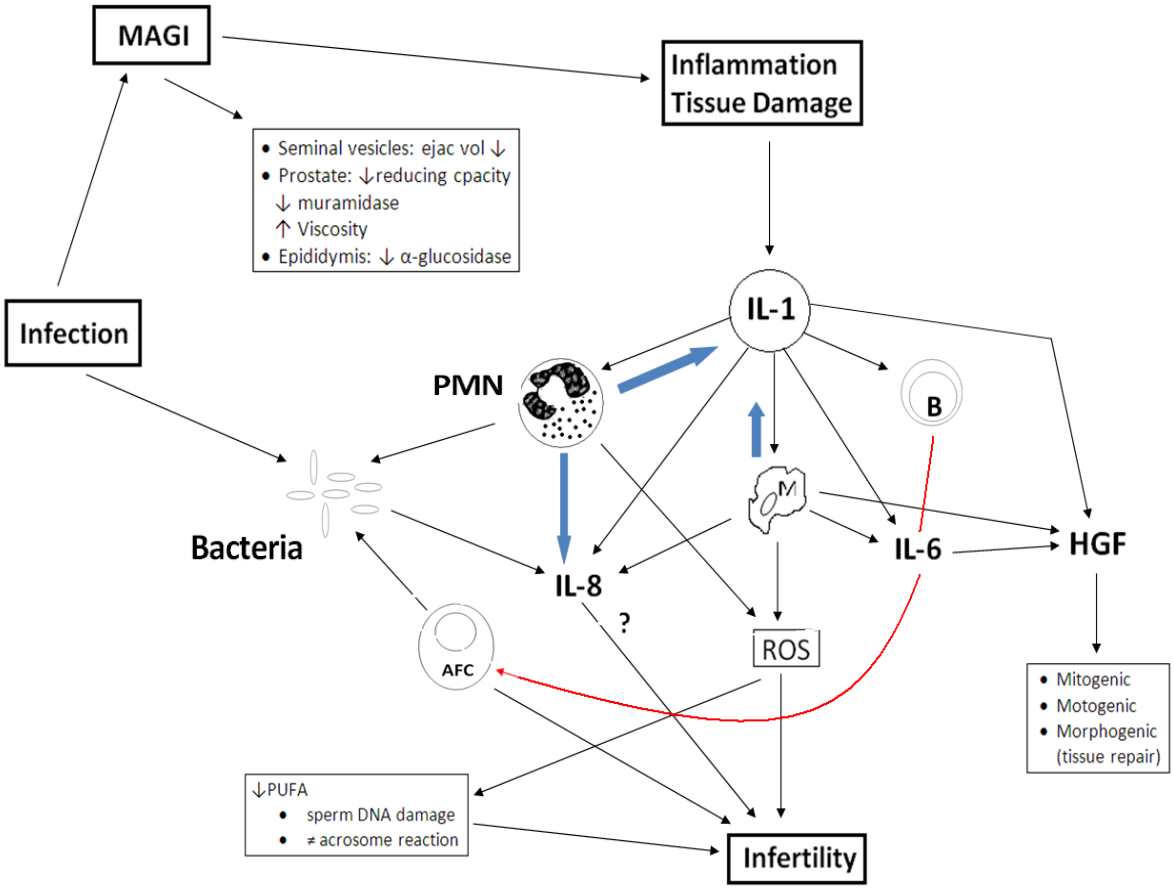


Figure 4.1: The effect of infection on the function of accessory glands (redrawn from Comhaire *et al.*, 1999). AFC = antibody-forming cell; B = B lymphocyte; HGF = hepatocyte growth factor; M = macrophage; PMN = polymorphonuclear white blood cell; PUFA = polyunsaturated fatty acids; ROS = reactive oxygen species.

IL-6 is an inflammatory trigger, thus starts the process. Also it causes leucocytes activation and differentiation. IL-6 participates to the final toxic effect through ROS overproduction. IL-8 participates to the neutrophil chemo-attraction phase in inflamed site and causes neutrophil activation and phagocytosis (Rajasekaran *et al*., 1995) and IL-6 mainly can cause membrane damage which lead to decrease spermatozoa functional capacity (Martinez-Prado, 2010). IL-8 is considered as a marker of MAGI and has a negative effect on fertilizing potential of spermatozoa (Depuydt *et al*., 1998). IL-8 is a reliable and prognostic marker (Castro *et al*., 2004; Penna *et al*., 2009) to diagnose of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) and benign prostatic hyperplasia (BPH).

In an *in vitro* study (Al-Mously and Eley, 2007) it was suggested that raised levels of IL-6 and IL-8 is a useful marker for upper genital tract infection especially prostatitis. Therefore, high levels IL-8 in the *C. trachomatis* infected patients suggest that the infection is more likely derived from upper genital tract (Al-Mously and Eley, 2007). *C. trachomatis* can induce the production of IL-8 by infected cells during *in vivo* and *in vitro* infection (Mpiga *et al*., 2006). IL-6 plays an important role in host defence against different organisms and *C. trachomatis* as well (Magee *et al* 1992; Ross *et al*., 2006). Elevated IL-6 and IL-8 levels have been observed in persistent and chronic *C. trachomatis* infection (Mpiga *et al*., 2006). High seminal plasma IL-6 and IL-8 were associated with *C. trachomatis* infection (Kokab *et al*., 2010; Eggert-Kruse *et al*., 2001). Although Eggert-Kruse *et al* (2003) stated that the role of *C. trachomatis* is unclear and diagnosis test like PCR and serological test does not provide reliable results. They also mentioned IL-8 and IL-6 are correlated with the number of leucocytes. IL-6 is the most important cytokines in inflammatory disease of seminal tract. Comhaire *et al*., (1994) first reported IL-6 levels in the seminal plasma that were higher in the infertile patients with leucocytospermia. In addition IL-8 titre was found higher in seminal plasma of infertile patients with leucocytospermia, also there is correlation between levels of IL-8 and IL-6 in seminal plasma (Shimoya *et al*., 1995).

There is controversy whether certain pro-inflammatory cytokines (such as IL-6 and IL-8 which are involved in inflammatory processes) in infertile patients are related to semen quality (Eggert-Kruse *et al*., 2001; Kopa *et al*., 2005). Previous studies suggested that there were no relationship (Comhaire *et al*., 1994; Matalliotakis *et al*., 2002), whereas more recent studies have supported such a relationship (Furuya *et al*., 2003; Kopa *et al*., 2005).

A positive correlation was found between IL-8 and seminal leucocytes (Eggert-Kruse *et al*., 2001) and Kokab *et al* (2010) found that high levels of IL-8 related to high semen volume that supported the previous study which showed *C. trachomatis* infected patients have increased semen volume (Hosseinzadeh *et al*., 2004). Bezold *et al*., (2007) found there is no relationship between pathogen DNA in semen and inflammatory markers. Also interleukins concentrations are not related to the presence of wide range bacteria in semen samples (Eggert-Kruse *et al*., 2001).

This Chapter therefore tests the general hypothesis that *C. trachomatis* infection is related to the levels of seminal inflammatory markers and these will also correlate with semen quality.

4.2 Materials and Methods

In this chapter the *C. trachomatis* prevalence data from Chapter 3 (PCR and serology results) was used to compared to data obtained from semen analysis performed in Yazd (see section 2.2.2) and measurements of semen inflammatory markers performed in Sheffield (see section 2.3.8.1 & 2.3.8.2). These data were first analysed by histogram to confirm normality. According to the distribution histogram and normality plots test on all variables, parametric and non-parametric independent t test were performed on normal and abnormal distributed data respectively as shown in the footnote of each table. Then Descriptive statistics and an independent sample T test were used for data with normal distribution and Mann-Whitney U test was used for nan- parametric data. Spearman rank correlation test was used to evaluate the relationship between the levels of IL-6 and IL-8 with semen parameters (see section 2.5).

4.3 Results

4.3.1 *C. trachomatis* infection and semen parameters

Comparisons were made between *C. trachomatis*-infected and uninfected men as defined by the presence of IgM and IgG antibodies in serum (see section 2.3.1) or *C. trachomatis* DNA in urine and their corresponding semen parameters evaluated according to WHO (1999) guidelines.

Table 4.1 shows the relationship between mean (±SD) age, duration of infertility, semen parameters of three IgM positive men and 247 IgM negative men. In summary there was no significant difference between the two groups and any variable measured. Since only three men were positive for IgM, the comparison with IgM negative men is not statistically robust.

Table 4.2 shows the relationship between mean (±SD) age, duration of infertility, semen parameters in *C. trachomatis* infected and uninfected men according to the PCR of urine DNA. Eleven men were positive for *C. trachomatis* by this method. Statistical analysis showed that only semen volume was significantly lower in *C. trachomatis* infected men (P= 0.001).

Table 4.3 shows the relationship between mean (±SD) age, duration of infertility, semen parameters in the IgG positive and IgG negative men. Forty five men were IgG positive and 205 were IgG negative. The semen pH was higher in the IgG (+) group compared with the IgG negative group and was biological significant (P=0.055, $0.05<P<0.1$). Also the percentage of immotile sperm was significantly lower in the IgG positive group compared with the IgG negative group (P= 0.018).

In each case anti-sperm antibodies (IgA & IgG) were tested for all individuals (see section 2.2.2). However the kits did not work properly, presumably because of shipping difficulties to Iran which may have led to them being stored inappropriately.

Table 4.1: Age, duration of infertility, semen parameters (mean ± SD) in *C. trachomatis* IgM (+) and IgM (-) men.

Variable	IgM Positive (n= 3)	IgM Negative (n= 247)	P-value
Age[a] (years)	**29.66 ± 3.51**	**32.76 ± 5.14**	**P=0.354**
Duration of infertility[a] (years)	**2.30 ± 0.57**	**6.00 ± 3.52**	**P=0.072**
Semen volume[a] (ml)	**2.00 ± 0.50**	**3.11 ± 1.52**	**P=0.078**
pH[a]	**8.33 ± 0.76**	**8.20 ± 0.43**	**P=0.150**
Sperm concentration[a] million/ml	**35.66 ± 39.71**	**66.51 ± 51.46**	**P=0.657**
Percent progressive Motile[b]	**25.00 ± 25.05**	**52.24 ± 20.97**	**P=0.059**
Percent immotile[b]	**35.66 ± 34.50**	**34.52 ± 19.63**	**P=0.812**
Percent normal morphology[b]	**6.30 ± 8.50**	**6.06 ± 3.51**	**P=0.579**
Percent DNA fragmentation[b]	**8.66 ± 10.66**	**12.69 ± 8.25**	**P=0.407**
Leucocytes[a], million/ml	**0.95 ± 0.43**	**1.56 ± 1.34**	**P=0.279**

a: parametric independent t test; b: Mann-Whitney U test

Table 4.2: Age, duration of infertility, semen parameters and (mean ± SD) in *C. trachomatis* urine DNA (+) and urine DNA (-) men.

Variable	DNA positive (n= 11)	DNA negative (n= 239)	P-value
Age[a] (years)	**31.45 ± 3.53**	**32.79 ± 5.19**	**P=0.254**
Duration of infertility[a] (years)	**5.27 ± 3.06**	**5.90 ± 3.49**	**P=0.406**
Semen volume[a] (ml)	**2.11 ± 0.73**	**3.15 ± 1.53**	**P=0.001***
pH[a]	**8.22 ± 0.41**	**8.25 ± 0.43**	**P=0.829**
Sperm concentration[a] million/ml	**59.54 ± 45.22**	**66.45 ± 51.27**	**P=0.632**
Percent progressive Motile[b]	**53.63 ± 15.71**	**51.52 ± 21.86**	**P= 0.781**
Percent immotile[b]	**33.36 ± 14.86**	**34.59 ± 19.98**	**P=0.923**
Percent normal morphology[b]	**7.27 ± 3.45**	**6. 08 ± 3.53**	**P=0.250**
Percent DNA fragmentation[b]	**11.18 ± 5.94**	**12.82 ± 8.31**	**P=0.673**
Leucocytes[a], million/ml	**1.60 ± 1.18**	**1.55 ± 1.34**	**P=0.882**

a: parametric independent t test; b: Mann-Whitney U test

*P-value≤ 0.05

Table 4.3: Age, duration of infertility, semen parameters (mean ± SD) in *C. trachomatis* IgG (+) and IgG (-) men.

Variable	IgG Positive (n= 45)	IgG Negative (n=205)	P-value
Age[a] (years)	**32.00 ± 5.20**	**32.9 ± 5.10**	**P=0.273**
Duration of infertility[a] (year)	**5.33 ± 3.04**	**6.09 ± 3.61**	**P=0.175**
Semen volume[a] (ml)	**3.30 ± 1.90**	**3.00 ± 1.40**	**P=0.748**
pH[a]	**8.40 ± 0.36**	**8.20 ± 0.44**	**P=0.055¶**
Sperm concentration[a] million/ml	**64.65 ± 42.3**	**68.54 ± 54.9**	**P=0.915**
Percent progressive motile[b]	**56.84 ± 21.4**	**52.23 ± 19.50**	**P=0.697**
Percent immotile[b]	**31.05 ± 19.50**	**36.20 ± 18.50**	**P=0.018***
Percent normal morphology[b]	**5.60 ± 2.60**	**6.20 ± 3.70**	**P=0.672**
Percent DNA fragmentation[b]	**12.20 ± 8.30**	**12.7 ± 8.00**	**P=0.592**
Leucocytes[a], million/ml	**0.98 ± 0.49**	**1.65 ± 1.41**	**P=0.152**

a: parametric independent t test; b: Mann-Whitney U test

*P-value≤ 0.05

¶0.05<P<0.1- although not slightly significant statistically this deserves consideration as being biological significant.

4.3.2 Inflammatory markers in semen

4.3.2.1 IL-6 and IL-8 levels in seminal fluid

Comparisons were made between the *C. trachomatis*-infected and uninfected men and the corresponding IL-6 and IL-8 levels in their seminal fluids. The median concentration of IL-6 in the seminal fluid from the total 250 male partners of infertile couples in the present study was 23.65 pg/ml (range 0.23-367.52 pg/ml), whereas the median concentration of IL-8 was 1615.17 pg/ml (range 179.50-41918.62 pg/ml). The 25-75 centiles and ranges were determined for both cytokines and the results are shown in Table 4.4 - 4.9.

The results from Table 4.4 shows the levels of seminal IL-6 in the male partners with genital tract chlamydial infection, as defined by IgM antibody to *C. trachomatis* in serum. However, as only three men were positive for IgM, therefore the comparison with IgM negative men is not statistically robust.

Table 4.5 shows the levels of seminal IL-6 in men with and without *C. trachomatis* DNA in their urine. Eleven men were positive by this method and 239 were negative. The mean ± SD of IL-6 was numerically higher in *C. trachomatis* infected men, however, it was not statistically significant (P= 0.244).

Table 4.6 shows the levels of seminal IL-6 in the male partners with genital tract chlamydial infection as defined by the presence of IgG antibody to *C. trachomatis* in serum. Forty five men were positive for IgG antibody to *C. trachomatis*. Interestingly, the level of IL-6 (mean ± SD) was significantly higher in the IgG positive men (P= 0.056, $0.05<P<0.1$ was considered biological relevant).

Seminal plasma concentration of IL-6>37.9 pg/ml in an individual male, was defined as 'high' (based on the 75th percentile) and this level was detected in a total 62 of the 250 samples tested. 9 of 62 men had a history of urethritis. High levels of IL-6 was detected in 4 of 11 samples were defined positive by NPPCR (urine DNA) and 14 of 45 samples were defined positive by serology (IgG).

The results from Table 4.7 shows the levels of seminal IL-8 in the male partners with genital tract chlamydial infection as defined by the presence of IgM antibody to *C.*

trachomatis in serum. However, as only three men were positive for IgM, therefore the comparison is not statistically precise.

Table 4.8 shows the levels of seminal IL-8 in men with and without *C. trachomatis* DNA in their urine. Eleven men were positive by this method. The mean ± SD of IL-8 was numerically higher in *C. trachomatis* infected men, however, it was not statistically significant (P= 0.157).

Table 4.9 shows the levels of seminal IL-8 in the male partners with genital tract chlamydial infection as defined by the presence of IgG antibody to *C. trachomatis* in serum. Forty five men were positive for IgG antibody to *C. trachomatis*. The mean ± SD and median of IL-8 was numerically higher in *C. trachomatis* infected men but these were not statistically significant different where compared to men who were IgG negative (P=0.680).

Seminal plasma concentration of IL-8>2966 pg/ml in an individual male, was defined as 'high' (based on the 75th percentile) and this level was detected in a total 62 of the 250 samples tested. 5 of 62 men had a history of urethritis. High levels of IL-8 was detected in 3 of 11 samples were defined positive by NPPCR (urine DNA) and 9 of 45 samples were defined positive by serology (IgG).

4.3.2.2 Correlation between IL-6 and IL-8 levels

The values of IL-6 and IL-8 cytokine levels irrespective of *C. trachomatis* infection were correlated. Figure 4.2 shows a degree of correlation (Pearson's correlation coefficient=0.38; P<0.001) between these inflammatory cytokines in seminal plasma of male partners of infertile couples.

4.3.2.3 IL-6 and IL-8 levels and semen parameters

Semen parameters of the 250 male partners of infertile couples were then evaluated respect to the IL-6 and IL-8 levels in their seminal fluid. Table 4.10 shows the correlation between the levels of IL-6, IL-8 and age, duration of infertility and semen parameters. IL-6 and IL-8 both were correlated significantly with the number of leucocytes in semen (P=0.012; P=0.001 respectively). Also IL-8 was inversely correlated with semen volume (P=0.013) and positively with male age (P=0.039). These results are shown graphically in Figures 4.3-4.6.

Table 4.4: Relationship between the levels of IL-6 with genital chlamydial infection (IgM) in male partners of infertile couples.

IL-6			
	Chlamydia infected (n= 3)	**Chlamydia uninfected (n= 247)**	**Total (n=250)**
Mean ± SD pg/ml	**32.61 ± 6.69**	**34.67 ± 47.42**	**34.64 ± 47.13**
Median pg/ml	**30.16**	**23.64**	**23.65**
25^{th}-75^{th} percentile pg/ml	**n/a**	**10.26-37.82**	**10.26-37.91**
Range pg/ml	**27.50-40.19**	**0.23-367.52**	**0.23-367.52**

Table 4.5: Relationship between the levels of IL-6 with genital chlamydial infection (urine DNA) in male partners of infertile couples.

IL-6			
	Chlamydia infected (n= 11)	**Chlamydia uninfected (n= 239)**	**Total (n=250)**
Mean ± SD pg/ml	**70.89 ± 101.19**	**32.97 ± 42.78**	**34.64 ± 47.13**
Median pg/ml	**28.28**	**23.64**	**23.65**
25^{th}-75^{th} percentile pg/ml	**20.23-72.62**	**10.05-37.82**	**10.26-37.91**
Range pg/ml	**0.51-327.43**	**0.23-367.52**	**0.23-367.52**

Table 4.6: Relationship between the levels of IL-6 with genital chlamydial infection (IgG) in male partners of infertile couples.

IL-6			
	Chlamydia infected (n= 45)	**Chlamydia uninfected (n= 205)**	**Total (n=250)**
Mean ± SD pg/ml	**54.54 ± 81.46**	**31.65 ± 34.36**	**34.64 ± 47.13**
Median pg/ml	**29.42**	**23.25**	**23.65**
25^{th}-75^{th} percentile pg/ml	**11.56-55.79**	**10.16-37.26**	**10.26-37.91**
Range pg/ml	**0.24-367.52**	**0.23-270.03**	**0.23-367.52**

Table 4.7: Relationship between the levels of IL-8 with genital chlamydial infection (IgM) in male partners of infertile couples.

IL-8			
	Chlamydia infected (n= 3)	**Chlamydia uninfected (n= 247)**	**Total (n=250)**
Mean± SD pg/ml	**2765.45 ± 764.29**	**3166.81 ± 5477.84**	**3162.00 ± 5445.32**
Median pg/ml	**2457.96**	**1588.62**	**1615.17**
25^{th}-75^{th} percentile pg/ml	**n/a**	**1003.44-2964.41**	**1005.50-2966.20**
Range pg/ml	**2222.03-3616.67**	**179.5-41918.62**	**179.50-41918.62**

Table 4.8: Relationship between the levels of IL-8 with genital chlamydial infection (urine DNA) in male partners of infertile couples.

IL-8			
	Chlamydia infected (n= 11)	**Chlamydia uninfected (n= 239)**	**Total Men=250**
Mean± SD pg/ml	**9540.69 ± 14434.28**	**2868.42 ± 4505.63**	**3162.00 ± 5445.32**
Median pg/ml	**1524.46**	**1622.21**	**1615.17**
25^{th}-75^{th} percentile pg/ml	**982.10-15242.20**	**1006.18-2849.43**	**1005.50-2966.20**
Range pg/ml	**463.71-37668.35**	**179.50-41918.62**	**179.50-41918.62**

Table 4.9: Relationship between the levels of IL-8 with genital chlamydial infection (IgG) in male partners of infertile couples.

IL-8			
	Chlamydia infected (n= 45)	**Chlamydia uninfected (n= 205)**	**Total (n=250)**
Mean± SD pg/ml	**3926.82 ± 7670.52**	**2981.58 ± 4831.22**	**3162.00 ± 5445.32**
Median pg/ml	**1819.92**	**1588.62**	**1615.17**
25^{th}-75^{th} percentile pg/ml	**1024.95-3023.99**	**1004.81-2968-19**	**1005.50-2966.20**
Range pg/ml	**185.75-37668.35**	**179.50-41918.62**	**179.50-41918.62**

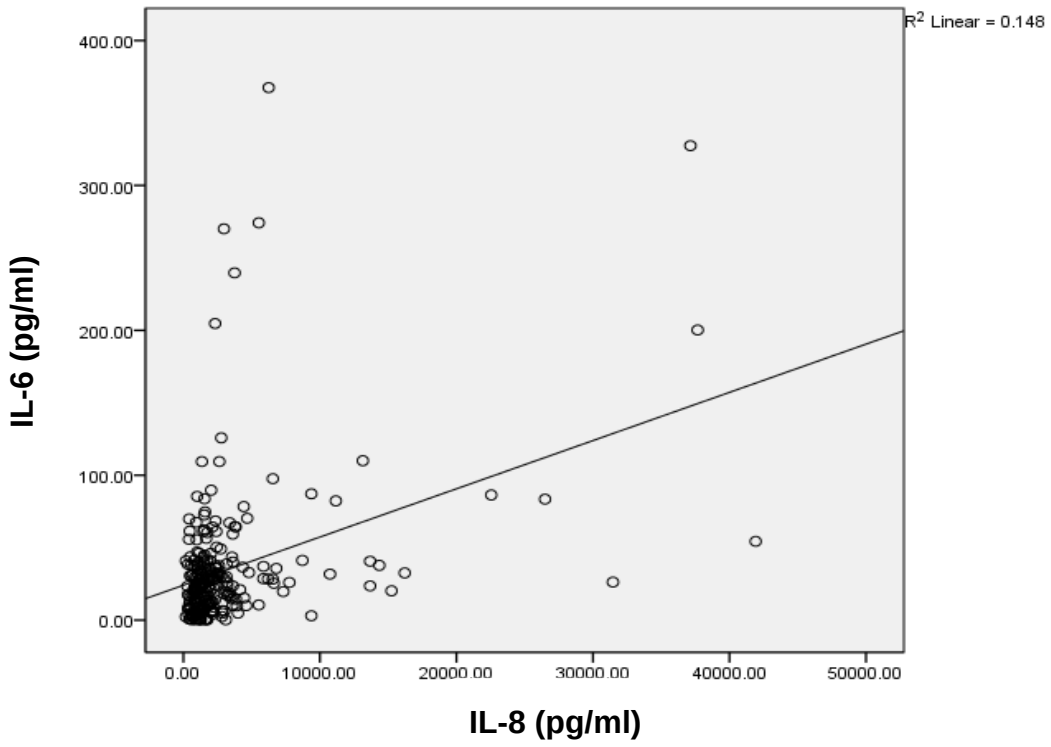


Figure 4.2: Correlation between IL-6 and IL-8 levels in seminal plasma of male partners of infertile couples (P<0.001).

Table 4.10: Median (range) of age, duration of infertility, semen parameters and their correlations with levels of IL-6 & IL-8 in semen.

Variable	Median(range)	Correlation with IL_6 levels	Correlation with IL_8 levels
Age (years)	**32.7 (21-52)**	**P=0.158 r=0.090**	**P=0.039* r=0.130**
Duration of infertility (years)	**5 (1-18)**	**P= 0.290 r= 0.068**	**p= 0.972 r= 0.002**
Semen volume (ml)	**2.5 (1-9.5)**	**P=0.175 r=0.086**	**P=0.013* r=-0.157**
pH	**8 (6-9.5)**	**P=0.672 r=0.022**	**P=0.241 r=0.074**
Sperm concentration million/ml	**62 (0.5-350)**	**P=0.326 r=0.062**	**P=0.383 r=0.055**
Percent progressive motile	**55 (0-95)**	**P=0.835 r=0.013**	**P=0.394 r=0.055**
Percent immotile	**32 (0-100)**	**P=0.914 r=0.007**	**P=0.882 r=0.010**
Percent normal morphology	**6 (0.5-17.5)**	**P=0.857 r=-0.012**	**P=0.775 r=0.018**
Percent DNA fragmentation	**11 (1-40)**	**P=0.975 r=0.002**	**P=0.951 r=0.004**
Leucocytes, million/ml	**1.2 (0.05-11.41)**	**P=0.012* r=0.159**	**P=0.001* r=0.419**

*P-value ≤ 0.05

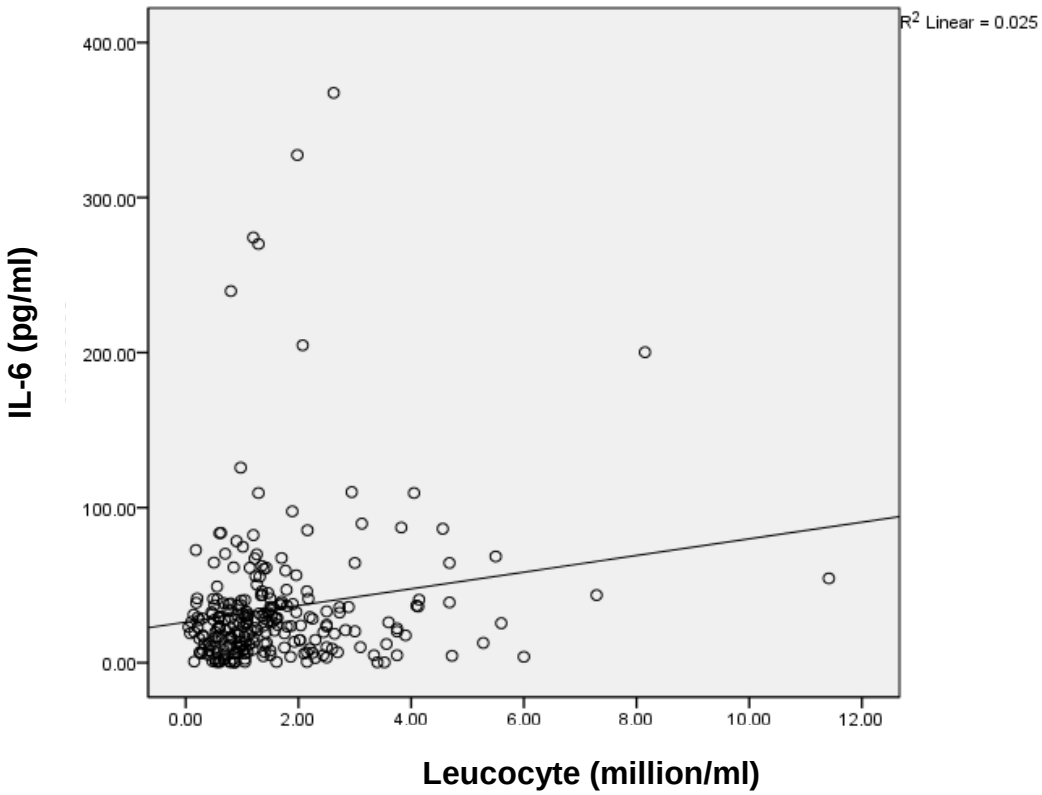


Figure 4.3: Correlation between concentration of IL-6 (pg/ml) and number of leucocytes (million/ml) in seminal plasma of male partners of infertile couples ($P<0.05$).

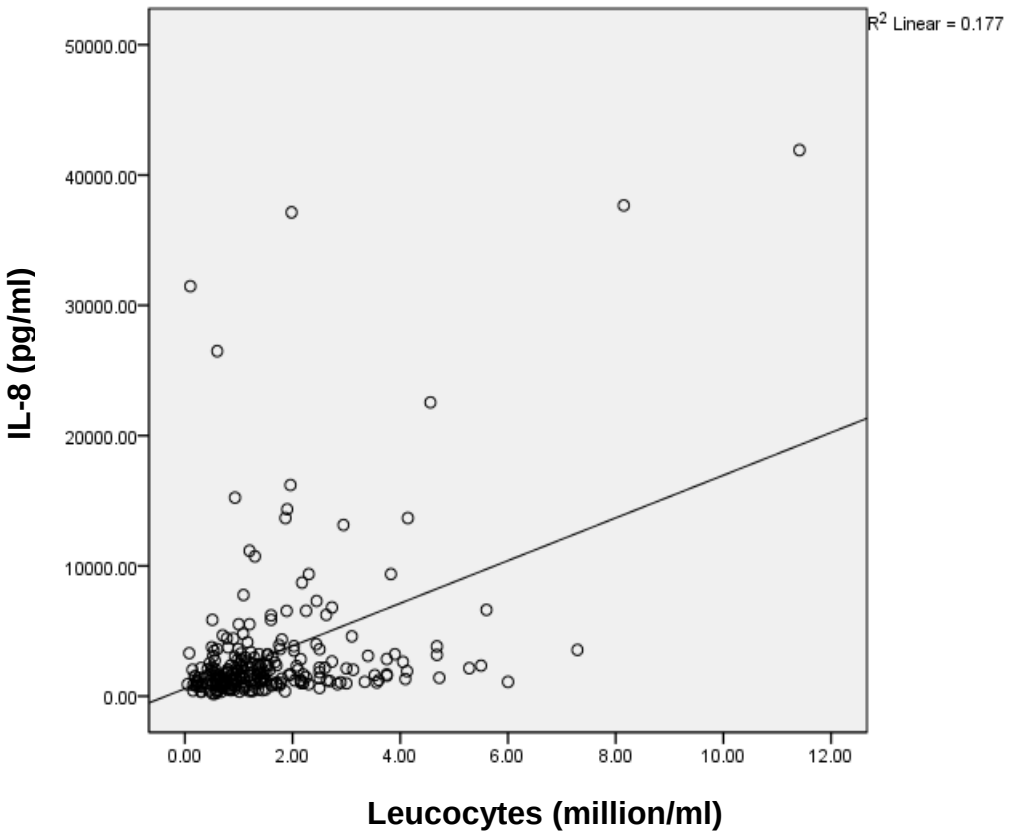


Figure 4.4: Correlation between concentration of IL-8 (pg/ml) and number of leucocytes (million/ml) in seminal plasma of male partners of infertile couples ($P<0.05$).

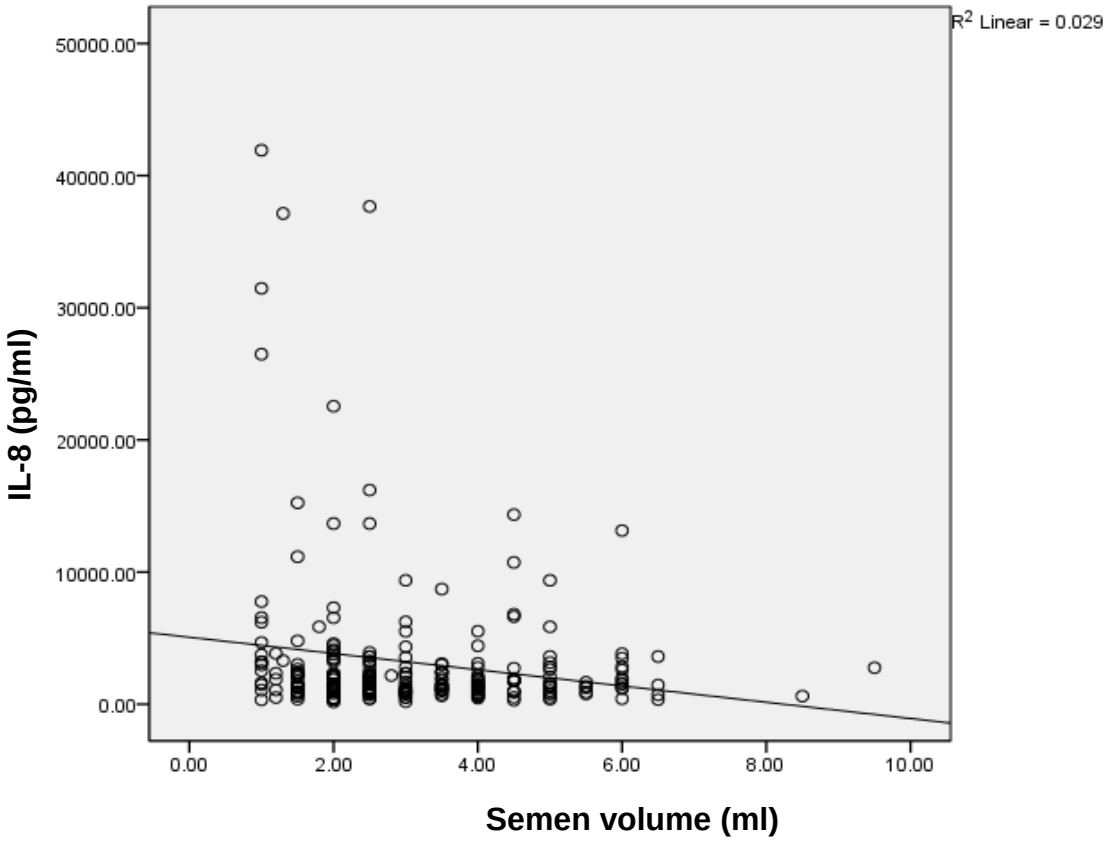


Figure 4.5: Correlation between concentration of IL-8 (pg/ml) and semen volume (ml) in male partners of infertile couples ($P<0.05$).

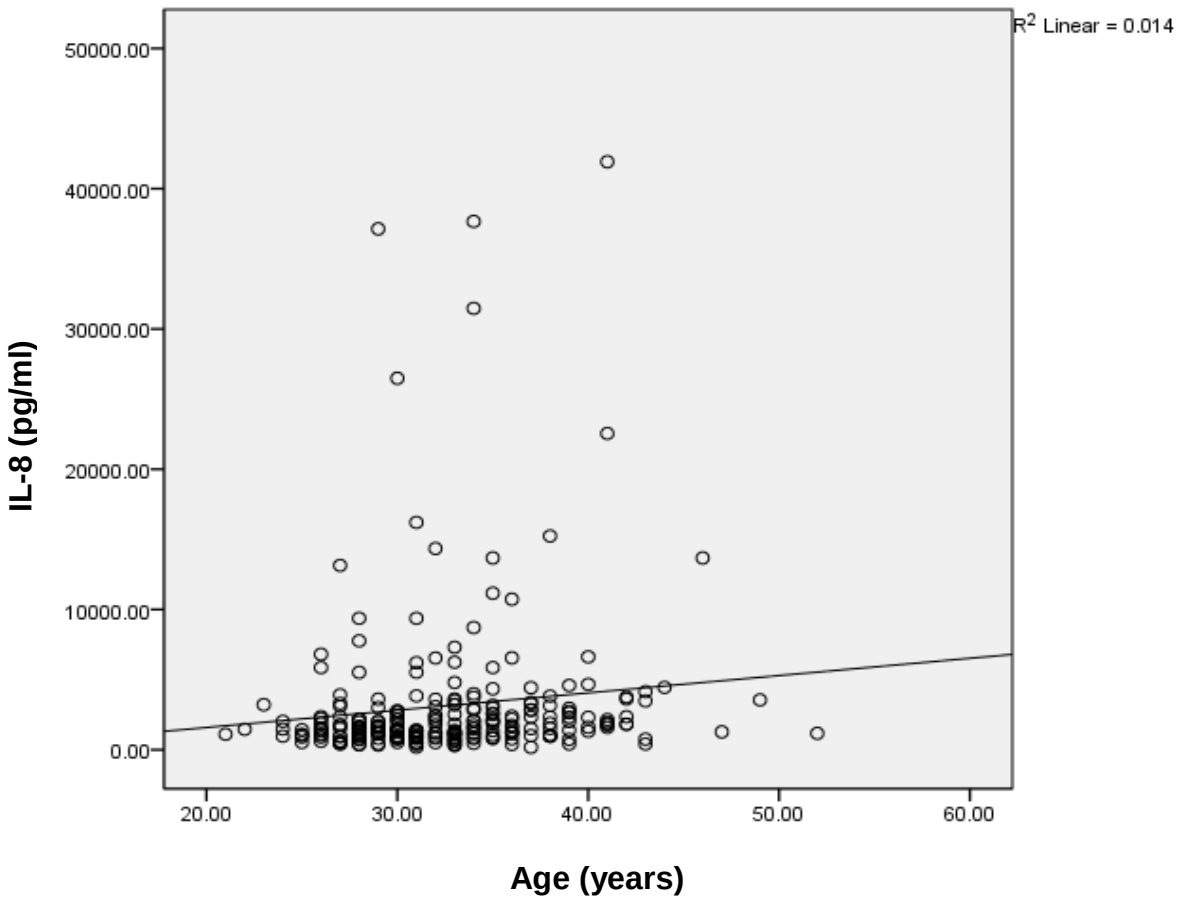


Figure 4.6: Correlation between concentration of IL-8 (pg/ml) and age of male partners (years) of infertile couples (P<0.05).

4.4 Discussion

This chapter examined the relationship between *C. trachomatis* infection in infected men (using PCR and serology) and inflammatory markers (IL-6 & IL-8) in the seminal plasma. The main conclusions are: (i) elevated IL-6 and IL-8 are observed in *C. trachomatis* positive men but this is not significant and it varies by diagnostic method; and (ii) IL-6 and IL-8 levels were correlated with each other and the concentration of leucocytes, but IL-8 was correlated with volume and age. These are discussed in more detail below.

The relationship between semen parameters and chlamydia infection in this study showed that men with such an infection in FVU samples (PCR positive) had only lower semen volume compared with men without infection. This is because inflammation can cause of obstruction of the duct and results to reduce semen volume and this support previous works (Wolff *et al*., 1991) and in contrast with others (Hosseinzadeh *et al*., 2004; Al-Mously *et al*., 2009; Kokab *et al*., 2010) who explained *C. trachomatis* infection might be cause of increased activity of accessory gland or reproductive epithelium secretion.

Infected men as defined by serology (IgG+) had high level of IL-6 and increased semen pH as described by Marconi *et al*., (2009) and Kokab *et al*., (2010).

The seminal plasma IL-6 level were much lower than IL-8 (0.23-367.52 pg/ml versus 179.50-41918.62 pg/ml) but significantly correlated to each other. These findings support the idea of Al-Mously and Eley (2007) who proposed that IL-6 and IL-8 seminal plasma levels might be useful as a marker of upper genital tract infection (e.g. prostatitis). Given that IL-6 and IL-8 are both markers of prostate inflammation and IL-8 is also related to infection of urethra (Al-Mously and Eley, 2007) the results might suggest that patients with high levels of ILs are more likely to have prostatitis and this is confirmed with the positive correlation of IL-8 with male age. Both ILs were also associated with high number of leucocytes in semen and this suggest the probability of inflammation and/or infection and this is similar to previous studies (Eggert-Kruse *et al*., 2001; Kokab *et al*., 2010).

Increasing IL-8 levels were associated with lower semen volume in *C. trachomatis* infected men (urine DNA+) and this is contrary to previous observations that found high

semen volume in infected men (Hosseinzadeh *et al*., 2004; Al-Mously *et al*., 2009; Kokab *et al*., 2010). It is unclear why this is the case although a simple explanation is that semen DNA was not infected in the present study and some men had only a history of urethritis (see Chapter 6). It might also be caused by unequal abstinence periods between the two groups, although this seems unlikely. IL-8 was also associated directly with male age in the present study, and this might be a sign of genital accessory gland infection (Al-Mously and Eley, 2007) or benign prostatic hyperplasia (BPH) without damage to spermatogenesis. There are studies about the role of IL-8 to trigger BPH (Berry *et al*., 1984; Castro *et al*., 2004; Penna *et al*., 2009) and developing BPH and male age (Berry *et al*., 1984; Glynn *et al*., 1984; Meigs *et al*., 2001). Since there is no correlation between age and semen volume were observed in the current study, the correlation between IL-8 and semen volume must be caused by accessory gland inflammation and/or infection by *C. trachomatis* or other infectious agent and the obstruction of the ductus epididymis (Marconi *et al*., 2009). The fact that the other microorganisms in semen are responsible for altered cytokines levels might be a complicating factor in interpreting these results.

These finding do not confirm previous studies on male infertility and *C. trachomatis* which have been carried out in Sheffield that found increased semen volume in infected men (Hosseinzadeh *et al*., 2004; Kokab *et al*., 2010). This might be because these studies undertook *C. trachomatis* diagnosis on semen samples which was not achieved in this study (see Chapter 3). However, these do confirm and extend the previous finding by Eggert-Kruse *et al* (2001) who found high level of ILs in leucocytospermic samples and no association with bacterial colonization of semen samples.

Therefore these correlations confirmed that *C. trachomatis* infection can result in high level of seminal plasma interleukins and statistically significant high level of IL-6 might suggest the effect of this organism on prostate and support the previous work in Sheffield (Al-Mously and Eley, 2007). Low semen volume in *C. trachomatis* infected men defined by positive urine DNA indicate current infection and can be indirectly related to high level IL-8 and inflammation of the prostate. Given the relationship between *C. trachomatis* infection and chronic prostatitis (Ouzounova *et al*., 2010) indicates the probability of a risk factor with prostate cancer (Stasiewicz *et al*., 2012)

and should be examined in future studies. However there is not an accepted aetiological link and the view is highly controversial.

The weakness of this study is the lack of a fertile group of males as control to see the level of ILs and their relationship with *C. trachomatis*. Extensive attempts were made to recruit fertile male participants in number of locations. These locations included antenatal and vasectomy clinic, however, despite these attempts no fertile male participant volunteered. These problems have been recognized in other studies (Cohn *et al*., 2002; Stewart *et al*., 2009, Eiser *et al*., 2011) and it is suggested they are too concerned about their fertility or suspected infertility to volunteer. Also they may have experience of testicular cancer treatment or anxiety of cancer diagnosis.

In the following chapters, the effect of *C. trachomatis* on sperm DNA and fertilizing capacity under *in vivo* and *in vitro* conditions of conception will be examined.

5 Chapter 5: Correlation between sperm DNA fragmentation and sperm chromatin integrity with *C. trachomatis* in infertile men

5.1 Introduction

This chapter investigates the role of *C. trachomatis* infection on sperm DNA in male partners of infertile couples. A direct effect of *C. trachomatis* EBs on spermatozoa that can lead to infertility is premature sperm death (Hosseinzadeh *et al*., 2001) through an apoptosis mechanism which is induced by *C. trachomatis* lipopolysaccharide (LPS) (Eley *et al*., 2005a; 2005b). The mechanism as defined in different studies (Aitken, 1994; Gorga *et al.*, 2001; Eley *et al*., 2005b) involves excessive production of reactive oxygen species (ROS) in leucocytes which has been induced by *C. trachomatis* lipopolysaccharide and ROS is mediator of apoptosis (Eley *et al*., 2005b).

Patients with genitourinary infection by *C. trachomatis* and *M. genitalium* showed increased sperm DNA fragmentation in comparison with fertile controls (Gallegos *et al.*, 2008). This increase is proportionally greater than the influence on classical semen parameters and could result in a decreased fertility potential (Gallegos *et al.*, 2008). Elevated levels of sperm DNA fragmentation are related to morphological abnormalities (Benchaib *et al*., 2007) and it is believed that normal sperm morphology can be a valuable predictor of the fertilization rate if it is evaluated by strict criteria (Kruger *et al*., 1999; Van Waart *et al*,. 2001). Assessment of DNA integrity may be a valuable marker of fertility for both animals and men. High level of chromatin damage has negative impact on both natural and ART conception (Evenson *et al*., 1980; Larson *et al*, 2000).

Although experimentally induced *in vivo* DNA damage of sperm is not possible in humans, strong associations have been shown between paternal genome damage by chemotherapeutic agents and embryo development in animals (Fernandez-Gonzales *et al*., 2008). The importance of studying sperm DNA integrity has been further intensified by the growing concern about transmission of genetic disease through ICSI (Barroso *et al*., 2000; Agarwal, 2003). Therefore in this chapter the effects of *C. trachomatis* on sperm DNA integrity was evaluated by different tests to examine the percentage of sperm DNA abnormality in infected males of infertile couples. The hypothesis that high levels of sperm DNA abnormality would be related to *C. trachomatis* infection was tested.

5.2 Materials and Methods

Spermatozoa from male partners of infertile couples were examined for DNA fragmentation using slides prepared during semen analysis (see section 2.2.1). Four different tests were used to detect sperm DNA abnormalities. Briefly, in Yazd/Iran tests for the detection of residual histones (section 2.3.7.2), evaluation of protamination (section 2.3.7.3) and detection of sperm chromatin condensation anomalies (section 2.3.7.4) were performed. A further test to detect sperm single or double DNA strand breaks (section 2.3.7.1) was performed using the TUNEL assay on slides shipped to Sheffield.

5.3 Results

5.3.1 Sperm DNA measurements

Comparison were made between *C. trachomatis*-infected and uninfected men with respect to the percentage of sperm DNA fragmentation (TUNEL assay) and the percentage of sperm with chromatin abnormality using the different DNA integrity tests. The results are shown in Table 5.1-5.4.

Table 5.1 shows the result obtained using the TUNEL assay (Figure 5.1) for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of DNA fragmentation between *C. trchomatis* positive and negative men, regardless of whether this was diagnosed by IgM, PCR or IgG.

Table 5.2 shows the results obtained using AB staining (Figure 5.2) for the detection of residual histones for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of chromatin abnormality between *C. trchomatis* positive and negative men, regardless of whether this was diagnosed by PCR or IgG. Unfortunately no results were available for diagnoses made using IgM (see below).

Table 5.3 shows the results obtained using AO staining (Figure 5.3) for detection of sperm chromatin condensation anomalies for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of chromatin abnormality between *C. trachomatis* positive and

negative men, regardless of whether this was diagnosed by PCR or IgG. Again no results were available for diagnoses made using IgM (see below).

Table 5.4 shows the results obtained using CMA3 staining (Figure 5.4) for evaluation of protamination for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of chromatin abnormality between *C. trchomatis* positive and negative men, regardless of whether this was diagnosed by PCR or IgG. IgM results were unavailable.

The study measured IgM for all serum samples. However, sperm chromatin studies were not performed on all 250 men. The fixed slides have been prepared for a sub-sample of 180 men. The matched samples for the three different staining (AB, AO and CMA3) were only achieved for a sub-sample of 80 men and therefore did not include any of the IgM positives.

Table 5.1: Sperm DNA fragmentation (TUNEL assay) in *C. trachomatis* positive men for: (i) IgM; (ii) DNA and (iii) IgG.

(i)	Mean ± SD IgM+ (n=3)	Mean ± SD IgM− (n=247)	P-value
TUNEL	**8.66 ± 10.66**	**12.69 ± 8.25**	**P=0.407**

(ii)	Mean ± SD DNA+ (n=11)	Mean ± SD DNA− (n=239)	P-value
TUNEL	**11.18 ± 5.94**	**12.82 ± 8.31**	**P=0.673**

(iii)	Mean ± SD IgG+ (n=45)	Mean ± SD IgG− (n=205)	P-value
TUNEL	**12.21 ± 8.28**	**12.71± 8.01**	**P=0.592**

Table 5.2: Detection of residual histone (Aniline Blue) in *C. trachomatis* positive men for: (i) IgM; (ii) DNA and (iii) IgG.

(i)	Mean ± SD IgM+ (n=0)	Mean ± SD IgM− (n=80)	P-value
Aniline Blue	**n/a**	**29.81± 15.6**	**n/a**

(ii)	Mean ± SD DNA+ (n=11)	Mean ± SD DNA− (n=69)	P-value
Aniline Blue	**28.0 ± 15.2**	**30.10 ± 14.8**	**P=0.657**

(iii)	Mean ± SD IgG+(n=21)	Mean ± SD IgG− (n=59)	P-value
Aniline Blue	**28.0 ± 13.9**	**28.85 ± 15.4**	**P=0.689**

Table 5.3: Sperm chromatin condensation anomalies (AO) in *C. trachomatis* positive men for: (i) IgM; (ii) DNA and (iii) IgG.

(i)	Mean ± SD IgM+ (n=0)	Mean ± SD IgM− (n=80)	P-value
Acridine Orange	**n/a**	**35.68 ± 12.20**	**n/a**

(ii)	Mean ± SD DNA+ (n=11)	Mean ± SD DNA− (n=69)	P-value
Acridine Orange	**37.54 ± 7.90**	**35.42 ± 12.60**	**P=0.462**

(iii)	Mean ± SD IgG+ (n=17)	Mean ± SD IgG− (n=63)	P-value
Acridine Orange	**35.52 ± 12.90**	**35.71 ± 12.17**	**P=0.957**

Table 5.4: Evaluation of protamination (Chromomycine A3) in *C. trachomatis* positive men for: (i) IgM; (ii) DNA and (iii) IgG.

(i)	Mean ± SD IgM+ (n=0)	Mean ± SD IgM− (n=80)	P-value
CMA3	**n/a**	**39.87 ± 10.6**	**n/a**

(ii)	Mean ± SD DNA+ (n=11)	Mean ± SD DNA− (n=69)	P-value
CMA3	**35.0±8.2**	**39.80 ± 10.6**	**P=0.156**

(iii)	Mean ± SD IgG+ (n=31)	Mean ± SD IgG- (n=49)	P-value
CMA3	**38.54 ± 8.22**	**40.63 ± 12.3**	**P=0.299**

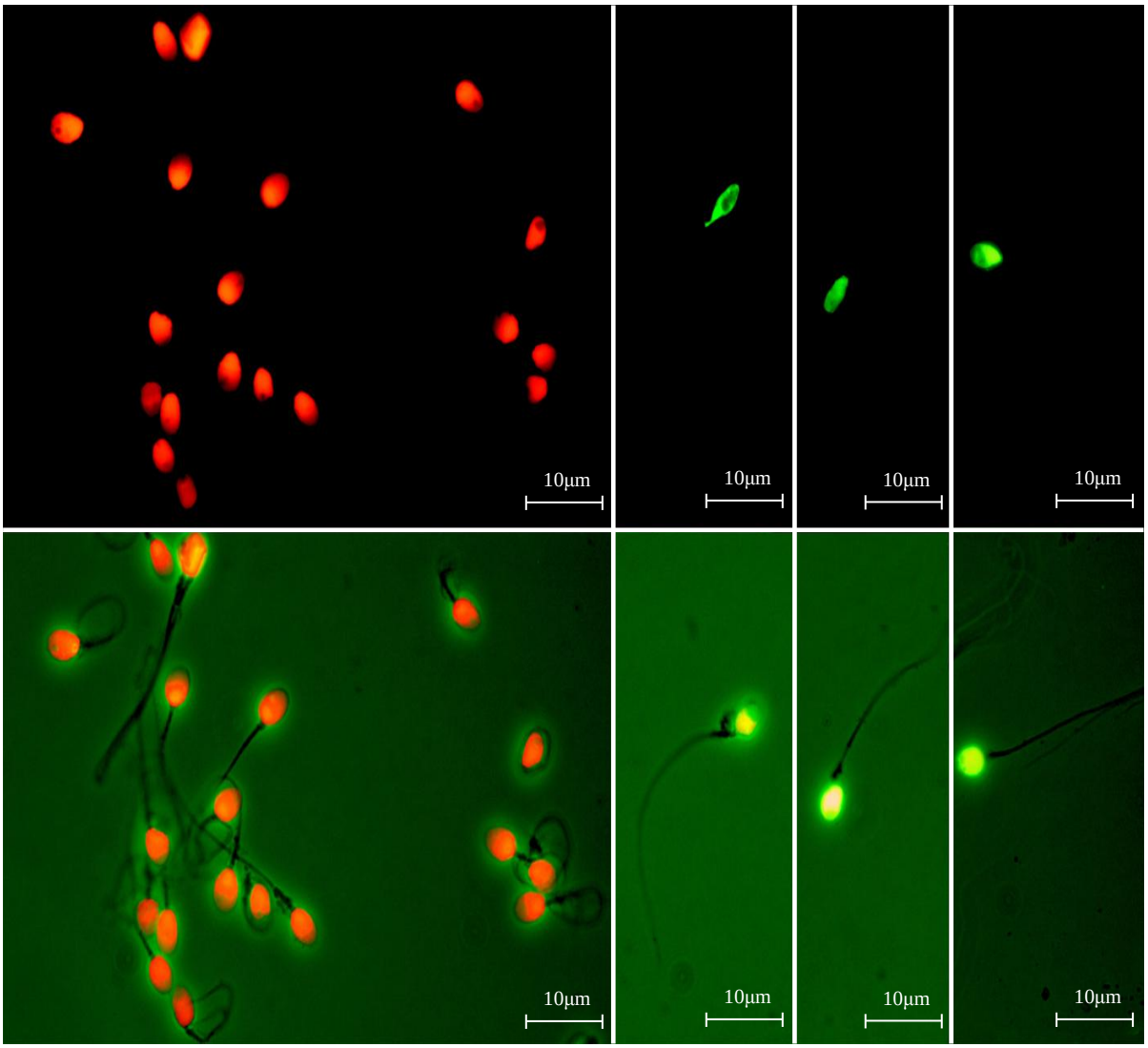


Figure 5.1: DNA fragmentation assay using TUNEL. Panels A & B show sperm with intact DNA, whereas C-H show sperm with damaged DNA. Upper panels show fluorescent signal and lower panels show corresponding image using visible light.

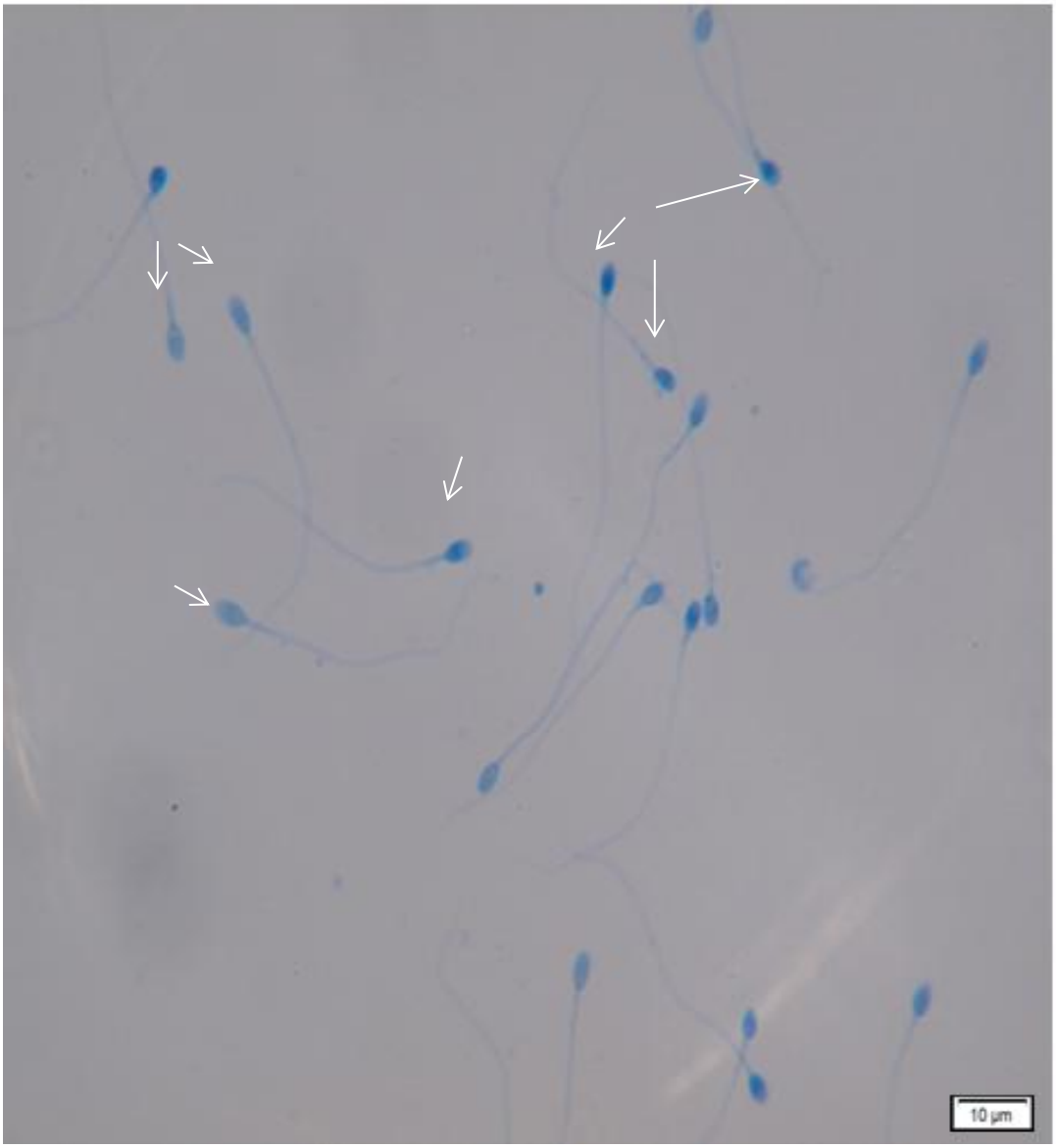


Figure 5.2: Sperm DNA integrity measurement using AB staining for detection of residual histones: (a) = abnormal spermatozoa (dark blue) and (b) = normal spermatozoa (pale blue or unstained).

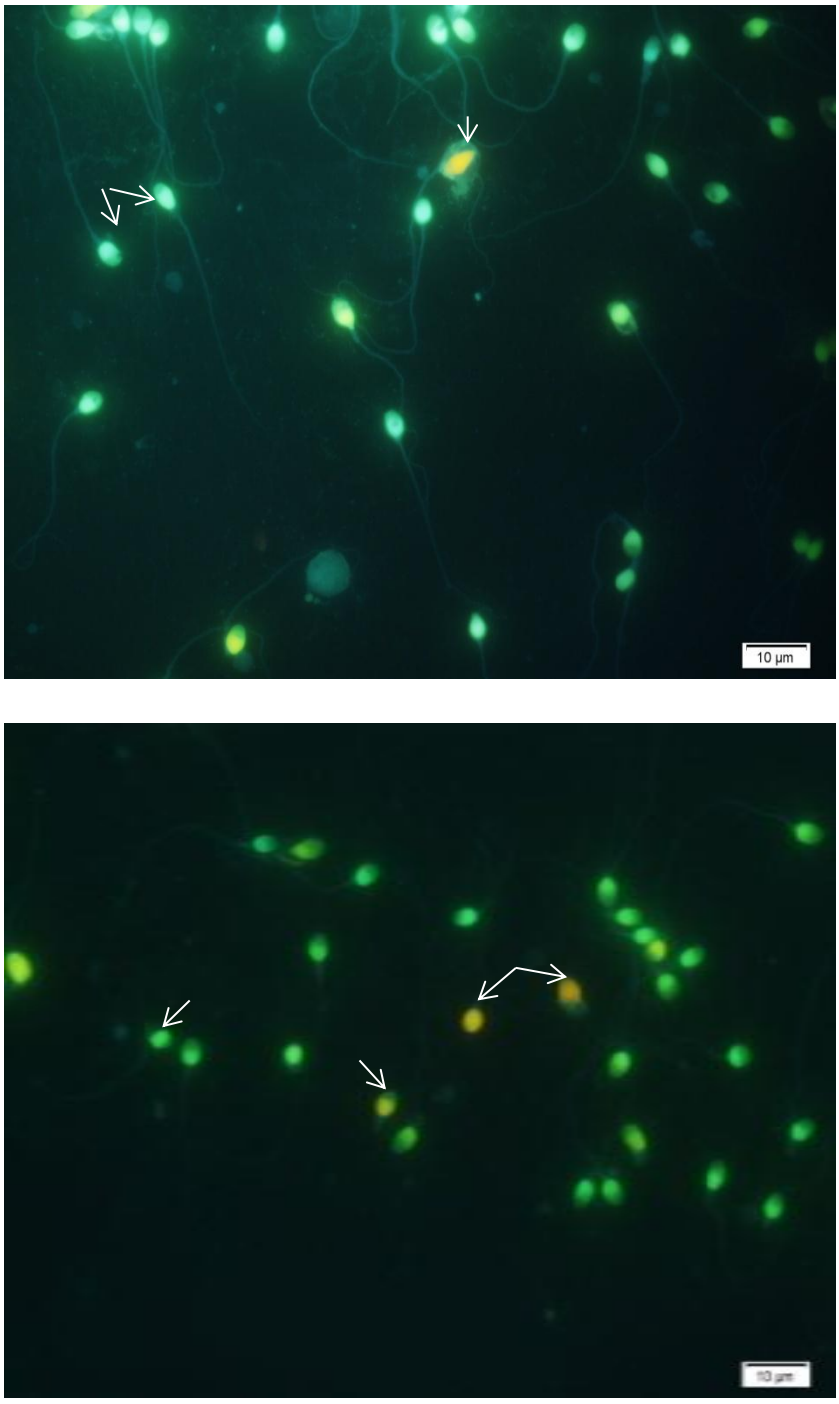


Figure 5.3: Sperm DNA integrity measurement using AO staining to detect sperm chromatin condensation anomalies: (a) = abnormal spermatozoa (orange-red; denatured DNA) and (b) = normal spermatozoa (green; double stranded DNA).

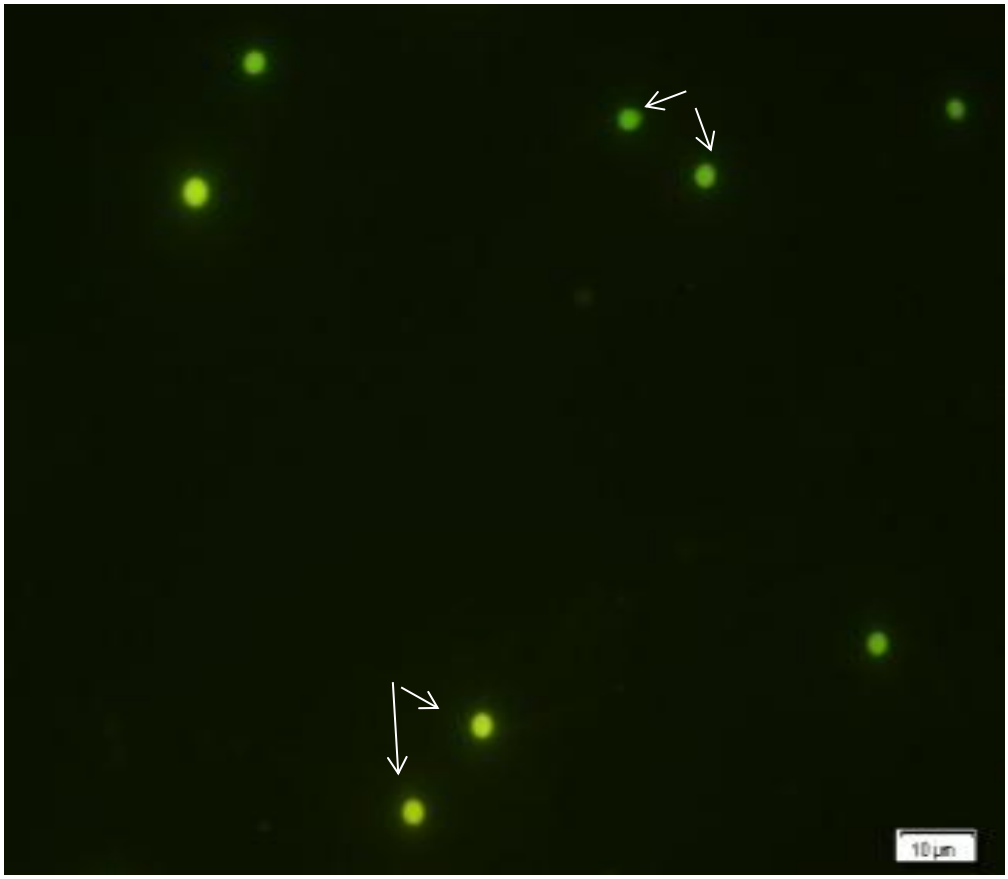


Figure 5.4: Sperm DNA integrity measurement using CMA3 staining for evaluation of protamination: (a) = abnormal spermatozoa (bright yellow) and (b) = normal spermatozoa (yellowish-green).

5.3.2 Semen parameters and sperm DNA fragmentation

Table 5.5 shows the relationship between DNA fragmentation using TUNEL assay and age, duration of infertility and semen parameters for the 250 male partners of infertile couples based the high (> 17%) and low (< 6%) level of DNA fragmentation as defined by 25^{th} and 75^{th} percentiles. Semen volume and the percentage of sperm with normal morphology were lower in the group of men with high percentage of sperm DNA fragmentation and this was statistically significant (P= 0.046, P= 0.003 respectively). In addition, percentage of immotile sperm was higher in this group and this was also statistically significant (P=0.044).

5.3.3 Semen parameters and sperm DNA integrity

Table 5.6 shows the correlation between three different tests used to measure sperm DNA integrity and age, duration of infertility and semen parameters of a sub-sample of 80 male partners of infertile couples for which these additional data were available. Briefly, the only significant correlation was seen between the percentage of CMA3 (evaluation of protamination) and male age (P=0.025). There was no correlation between age, duration of infertility and semen parameters with Aniline Blue staining (detection of residual histones) or Acridine Orange staining (sperm chromatin condensation anomalies).

5.4 Discussion

In this chapter, sperm DNA was assessed by different tests to examine whether it was related to *C. trachomatis* infection in the male partner of infertile couples. The main conclusions of the results are: (i) sperm DNA fragmentation (TUNEL) and chromatin studies (AB, AO & CMA3) were not correlated with *C. trachomatis* diagnosis; (ii) the percentage of DNA fragmentation is positively correlated with the percentage of immotile sperm but negatively with semen volume, normal morphology; and (iii) in sperm chromatin measurements only the percentage of protamination was related to male age.

Table 5.5: Age, duration of infertility, semen parameters (mean ± SD) in low and high levels of DNA fragmentation using TUNEL assay (based on 25th and 75th percentiles).

Variable	DNA fragmentation 25th percentile (n=64)	DNA fragmentation 75th percentile (n=67)	P-value
Age[a] (years)	**32.53 ± 4.51**	**32.22 ± 5.29**	**P=0.717**
Duration of infertility[a] (years)	**5.72 ± 2.85**	**6.27 ± 3.91**	**P=0.360**
Semen volume[a] (ml)	**3.48 ± 1.78**	**2.92 ± 1.48**	**P=0.046***
pH[a]	**8.26 ± 0.49**	**8.20 ± 0.42**	**P=0.843**
Sperm concentration[a] million/ml	**62.37 ± 66.82**	**68.13 ± 39.53**	**P=0.548**
Percent progressive Motile[b]	**45.63 ± 25.64**	**54.15 ± 18.96**	**P=0.094**
Percent immotile[b]	**33.12 ± 14.61**	**44.97 ± 28.57**	**P=0.044***
Percent normal morphology[b]	**6.95 ± 3.78**	**5.05 ± 3.45**	**P=0.003***
Leucocytes[a], million/ml	**1.35 ± 1.57**	**1.58 ± 1.29**	**P=0.349**

a: parametric independent t test; b: Mann-Whitney U test

*P-value≤ 0.05

Table 5.6: Age; duration of infertility; semen parameters (median) and their corresponding correlation with DNA integrity measured by Aniline Blue (AB), Acridine Orange (AO) and Chromomycine A3 (CMA3) for a sub-sample of 80 men.

Variable	Median(range) (n=80)	Correlation with %AB	Correlation with %AO	Correlation with %CMA3
Age (years)	**33 (22-49)**	**P=0.961 r=0.006**	**P=0.157 r=0.160**	**P=0.025* r=0.252**
Duration of infertility (years)	**4.3 (1-18)**	**P=0.765 r=0.034**	**P=0. 280 r=0.122**	**P=0.179 r=0.115**
Semen volume (ml)	**3.5 (1.0-9.5)**	**P=0.496 r=0.077**	**P=0.182 r=0.151**	**P=0.643 r=0.053**
pH	**8.5 (6.0-9.0)**	**P=0.897 r=0.015**	**P=0.857 r=0.020**	**P=0.203 r=0.145**
Sperm concentration million/ml	**74.0 (3.0-310.0)**	**P=0.124 r=0.173**	**P=0.982 r=0.003**	**P=0.466 r=0.083**
Percent progressive motile	**59.5 (0.0-95.0)**	**P=0.099 r=0.186**	**P=0.912 r=0.012**	**P=0.611 r=0.058**
Percent immotile	**32.0 (6.0-100.0)**	**P=0.574 r=0.064**	**P=0.447 r=0.086**	**P=0.422 r=0.092**
Percent normal morphology	**6.3 (0.5-14.5)**	**P=0.390 r=0.097**	**P=0.450 r=0.086**	**P=0.264 r=0.127**
Leucocytes, million/ml	**1.1(0.15-14.4)**	**P=0.092 r=0.192**	**P=0.818 r=0.027**	**P=0.061 r=0.215**

*P-value≤ 0.05

Sperm DNA fragmentation assessed by TUNEL assay was not related to *C. trachomatis* infection in the male partners of infertile couples as defined by serology (IgM & IgG) or PCR of urine samples. In addition to the results for DNA fragmentation, the results of this present study also showed *C. trachomatis* infection was not associated with various measures of sperm chromatin condensation such as AB, CMA3 and AO staining. This is in contrast with previous studies that showed sperm DNA fragmentation is higher in *C. trachomatis* infected infertile men (Satta *et al*., 2006; Gallegos *et al*., 2008). This is not a surprise because in this study semen samples were not infected by *C. trachomatis* using PCR which suggests there was no infection in the upper genital tract to affect spermatogenesis (see Chapter 3). The positive results for infection (urine DNA) indicate lower genital tract infection such as urethritis.

The results of TUNEL assay showed semen volume and percentage of normal morphology were significantly lower in infertile men with a "high" level of DNA fragmentation (based on 75^{th} percentiles) and the percentage of immotile sperm was significantly higher in "high" level of DNA fragmentation. These findings cannot be compared to other studies because as they used different methods including flow cytometry instead of assessing fixed slides and sperm chromatin investigation rather than double strand DNA break (TUNEL assay).

In this chapter sperm chromatin integrity was investigated by three methods and showed that the percentage of stained sperm was not directly associated with any semen variable. The results only suggested that the level of protamination as men get older is reduced compared to younger men. CMA3 competes with protamine in the same site of DNA (Plastira *et al*., 2007); therefore our results can be explained by age-related alteration of semen parameters that is similar to the work of Plastira *et al*., (2007) and their support of Wyrobek *et al*., (2006). Although Kidd *et al*., (2001) showed that increasing male age was associated with a decreasing in semen volume, sperm motility and morphology; they did not evaluate sperm DNA measurements. In contrast, Nijs *et al*., (2009) did not find any male age-related influences on routine semen parameters or sperm DNA measurements. There is an extensive literature about the relationship between semen parameters and sperm DNA damage (Zini *et al*., 2001; Shen *et al*., 2002; Saleh *et al*., 2002). For example, Zini and colleagues found DNA damage was negatively correlated with semen parameters and the strongest correlation was with

sperm motility (Zini *et al*., 2001), whereas Shen *et al*., (2002) found overall poor sperm quality in apoptotic samples. Saleh *et al*., (2002) have not found relationship between semen parameters and DNA fragmentation; however Saleh *et al*., (2002) found higher level of DNA fragmentation in infertile men compared to a fertile group. However, these investigations are between infertile men and DNA damage without taking into account *C. trachomatis* infection.

The limitations of this study were (i) lack of control group as this was not easy to get fertile men to take part in the study as discussed in Chapter four and (ii) sperm DNA chromatin assessment was performed only on a sub-sample of n=80 men and this did not include any of the IgM positive men and (iii) the method used for TUNEL assay was only assessing fixed slides and not flow cytometry which is more accurate. This is because flow cytometry was not available.

Implication of sperm DNA testing is controversial in infertility investigations. It might help couples with a history of long term unexplained infertility and ART failure (Sakkas & Alvarez, 2010). The American Society for Reproductive Medicine (ASRM) guideline (2013) suggested that although sperm DNA damaging is more common in infertile men, however, the clinical utility of testing is not recommended in routine infertility work-up, as these tests are not reliable to predict outcome of pregnancy (ASRM, 2013). In addition, due to lack of efficient treatment there is no more help for couples even if sperm DNA abnormalities were detected (ASRM, 2006). Also ASRM Practice Committee (2006) did not find relationship between sperm DNA damage and reproductive outcome either in spontaneous or assisted conception. Barratt *et al*., (2010) reported the results of an ESHRE workshop in 2009 which concluded sperm DNA abnormalities may have most effect on IUI pregnancy rates and pregnancy loss after IVF and ICSI. However, there are clinical and assay uncertainties because of the lack of a robust clinical test. The larger issues including: who to test, when to test and how to treat patients with abnormal results have not yet been resolved. To confirm the results of literatures, further larger, well designed and controlled prospective studies will be required (Barratt *et al*., 2010).

In the next chapter, *C. trachomatis* infection is considered with regard to pregnancy rate, pregnancy outcome of the 250 infertile couples.

6 Chapter 6: *C. trachomatis* infection, pregnancy rate, pregnancy outcome

6.1 Introduction

Infertility is one of the serious complications of *C. trachomatis* infection among women in reproductive age, due to the sequelae of PID following genital tract infection (Low *et al.*, 2006; Haggerty *et al*., 2010) that can lead to tubal factor infertility (TFI). In addition, semen parameters can be affected by *C. trachomatis* infection (Hosseinzadeh *et al*., 2000; 2001; 2003; 2004; Idahl *et al*., 2004; 2007) and this may also contribute to infertility. Therefore it might be hypothesized that the pregnancy rate and/or pregnancy outcome in infected couples is poorer than in those without infection.

With regard to conception, there are controversial studies showing the presence of *C. trachomatis* in female genital tract was associated with poor IVF outcome (Fanchin *et al*., 1998) and two Swedish studies by Idahl *et al*., (2004; 2007) showing that the *C. trachomatis* positive serology was negatively associated with semen parameters and lower pregnancy rate (unassisted) in infertile couples (Idahl *et al*., 2004; 2007). In contrast, de Barberyac *et al*., (2006) found in subfertile couples that *C. trachomatis* infection was not associated with semen parameters and pregnancy outcome by IVF (de Barberyac *et al*., 2006).

With regards to assisted pregnancy in infected men, as *C. trachomatis* is not removed from sperm by washing methods (Al-Mously *et al*., 2009) it might potentially result in contamination of IVF culture system and negatively affect embryo quality. Although, some microbes can be removed by sperm washing (Wong *et al*., 1986) and other may be sensitive to the antibiotics used in IVF culture media, *C. trachomatis* is not sensitive to all antibiotics that are added to culture media (Storey and Chopra, 2001; Pacey and Eley, 2004). Moreover, when pregnancy is established, *C. trachomatis* infection as an asymptomatic and persistent infection that can result in serious complication during pregnancy such as preterm premature rapture of membranes (PPROM), preterm birth (Gravett *et al*., 1986; Sweet *et al*., 1987) and ectopic pregnancy (Low *et al*., 2006; Haggerty *et al*., 2010).

In the present study the pregnancy rate, pregnancy outcome were investigated in the 250 infertile couples recruited to this study. As well as their *C. trachomatis* status, their past medical and reproductive history was taken into account in order to evaluate any relationship between these data and pregnancy rate and outcome.

6.2 Materials and Methods

In this chapter the past reproductive and general history of the 250 infertile couples enrolled to study was correlated with their *C. trachomatis* status defined by IgM, PCR and IgG. For this analysis infertility was divided into primary and secondary infertility. Primary infertility is defined as the lack of ability to conceive a child that results in a live birth after a year of unprotected coitus (Shaw, 2003). Whereas secondary infertility is defined as inability of a couple to conceive after a year or two of unprotected and appropriately timed intercourse when one or both partners have previously conceived children (Shaw, 2003). TFI was defined as the occlusion of one or both tubes as diagnosed either by laparoscopy and/or HSG (Joseph, 2011). Endometriosis was confirmed by laparoscopy using European Society of Human Reproduction and Embryology (ESHRE) guideline for diagnosis and treatment of endometriosis (Kennedy *et al*., 2005). PCOS was diagnosed by vaginal sonography and/or laparoscopy and considering hirsutism (hyperandrogenism) and oligo-amenorrhea in a general examination as described in the Rotterdam 2003 guideline (Fauser *et al*., 2004). The regular menstrual cycle was considered based on regular intervals. Miscarriage indicates loss an embryo or fetus before the 20th week of pregnancy, according to the definition published by Shaw (2003). The male partners were examined by a urologist and the data for any urogenital abnormality and varicocele was obtained from patient notes. Varicocele was diagnosed by exam and/or scan. Male factor infertility was defined based on semen parameters (WHO, 1999). Men who drove for more than 2 hours daily were classified as drivers.

In addition to the above, follow up data obtained 24 months after enrollment, was also correlated with *C. trachomatis* status. Follow up details included the outcome of any pregnancy (spontaneous or assisted) including live birth, still birth, miscarriage and ongoing pregnancy. Also the sex and weight of baby was born during follow up (see section 2.4). To explore confounding factors among the past medical histories of both partners logistic regression was used (see section 2.5).

6.3 Results

6.3.1 Past medical history and *C. trachomatis* prevalence in infertile men

Table 6.1-6.3 show the past history of urethritis, varicocelectomy, driving (>2 hours/day), smoking, opium addict and mumps as a function and *C. trachomatis* infection were assessed by serum IgM status (Table 6.1) PCR (Table 6.2) and serum IgG status (Table 6.3).

Briefly, no statistical correlations were seen between the incidences of either condition in relation to IgM antibody status (Table 6.1). But when these clinical data were examined with respect to *C. trachomatis* positivity by PCR of urine DNA (Table 6.2), it was found urethritis was more likely in infertile males who had *C. trachomatis* DNA in their urine (OR= 34.82; 95%CI= 8.45-143.40). This was not observed (Table 6.3) in men diagnosed by IgG status (OR= 1.50; 95%CI= 0.56–4.02). Among male partners of infertile couples, drivers were more likely to have exposure to *C. trachomatis* as defined by IgG (OR=2.37; 95%CI= 1.03-5.45), but no other variable were found to be significant.

6.3.2 Reproductive History and *C. trachomatis* prevalence in infertile women

Table 6.4-6.6 show the clinical data for the female partners, including type of infertility (primary or secondary), past history of miscarriage, use of ART, menstruation (regular/irregular), history of infection, PCOS, TFI and endometriosis which were examined for possible correlation with the presence of *C. trachomatis* infection as defined by serum IgM status (Table 6.4) urine PCR (Table 6.5) and serum IgG status (Table 6.6).

Briefly, only PCOS was statistically more common (OR= 3.70; 95%CI= 1.03-13.3) in relation to IgM antibody status (Table 6.4) but this was not observed (Table 6.5) in women diagnosed by PCR (urine DNA). However, when this was examined by IgG antibody status (Table 6.6) women with IgG antibody to chlamydia were 2.5, times more likely to have a past medical history of PCOS (OR= 2.59; 95%CI= 1.24-5.27).

Table 6.1: Past medical history and *C. trachomatis* prevalence (IgM) in infertile men.

Past Medical History		**No. (%)**	***C. trachomatis* infected No. (%)**	**Odds ratio**	**95% (CI)**
Urethritis	**Yes**	**25 (10.0)**	**0 (0.0)**	**3.08**	**0.31- 30.81**
	No	**225 (90.0)**	**3 (1.3)**		
Varicocelectomy	**Yes**	**61 (24.4)**	**1 (16.4)**	**1.55**	**0.14-17.49**
	No	**189 (75.6)**	**2 (1.05)**		
Driver	**Yes**	**32 (12.8)**	**1 (3.1)**	**3.48**	**0.31-39.56**
	No	**218 (87.2)**	**2 (0.91)**		
Smoker	**Yes**	**49 (19.6)**	**1 (2.04)**	**2.07**	**0.18-23.33**
	No	**201 (80.4)**	**2 (0.99)**		
Opium addict	**Yes**	**14 (5.6)**	**0 (0.0)**	**5.97**	**0.58-61.46**
	No	**236 (94.4)**	**3 (1.3)**		
Mumps	**Yes**	**5 (1.2)**	**0 (0.0)**	**8.96**	**0.39-206.2**
	No	**245 (98.8)**	**3 (1.2)**		

Table 6.2: Past medical history and *C. trachomatis* prevalence (urine DNA) in infertile men.

Past Medical History		No. (%)	*C. trachomatis* infected No.(%)	Odds ratio	95% (CI)
Urethritis	**Yes**	**25 (10.0)**	**8 (32.0)**	**34.82***	**8.45-143.40**
	No	**225 (90.0)**	**3 (1.3)**		
Varicocelectomy	**Yes**	**61 (24.4)**	**4 (6.5)**	**1.82**	**0.68-3.44**
	No	**189 (75.6)**	**7 (3.7)**		
Driver	**Yes**	**32 (12.8)**	**2 (6.3)**	**1.44**	**0.39-5.30**
	No	**218 (87.2)**	**9 (41.3)**		
Smoker	**Yes**	**49 (19.6)**	**4 (81.6)**	**1.93**	**0.85-4.40**
	No	**201 (80.4)**	**7 (3.5)**		
Opium addict	**Yes**	**14 (5.6)**	**0 (0.0)**	**0.75**	**0.04-13.59**
	No	**236 (94.4)**	**11 (4.6)**		
Mumps	**Yes**	**5 (1.2)**	**0 (0.0)**	**5.36**	**0.22-126.10**
	No	**245 (98.8)**	**11 (4.5)**		

*Significant odds ratio

Table 6.3: Past medical history and *C. trachomatis* prevalence (IgG) in infertile men.

Past Medical History		No. (%)	*C. trachomatis* infected No. (%)	Odds ratio	95% (CI)
Urethritis	Yes	25 (10.0)	6 (24.0)	1.50	0.56-4.02
	No	225 (90.0)	39 (17.3)		
Varicocelectomy	Yes	61 (24.4)	13 (21.3)	1.32	0.65-2.73
	No	189 (75.6)	32 (16.9)		
Driver	Yes	32 (12.8)	10 (31.2)	2.37*	1.03-5.45
	No	218 (87.2)	35 (16.0)		
Smoker	Yes	49 (19.6)	5 (10.2)	0.45	0.17-1.23
	No	201 (80.4)	40 (19.9)		
Opium addict	Yes	14 (5.6)	2 (14.3)	0.74	0.16-3.46
	No	236 (94.4)	43 (18.2)		
Mumps	Yes	5 (2.0)	1 (20.0)	1.14	0.12-10.47
	No	245 (98.0)	44 (17.9)		

*significant odds ratio

Table 6.4: Reproductive history and *C. trachomatis* prevalence (IgM) in infertile women.

Reproductive history		No. (%)	*C. trachomatis* infected No. (%)	Odds ratio	95% (CI)
Infertility	**Primary**	**182 (73.0)**	**8 (4.4)**	**1.51**	**0.31-7.33**
	Secondary	**68 (27.2)**	**2 (2.9)**		
Miscarriage	**Yes**	**41 (16.4)**	**2 (2.4)**	**1.28**	**0.26-6.29**
	No	**209 (83.6)**	**8 (3.8)**		
ART	**Yes**	**54 (21.6)**	**2 (3.7)**	**0.90**	**0.18-4.39**
	No	**196 (78.4)**	**8 (4.0)**		
Menstruation	**Regular**	**164 (65.6)**	**6 (3.6)**	**0.77**	**0.21-2.84**
	Irregular	**86 (34.4)**	**4 (4.6)**		
Infection	**Yes**	**137 (54.8)**	**6 (4.4)**	**1.24**	**0.34-4.53**
	No	**113 (45.2)**	**4 (3.5)**		
PCOS	**Yes**	**56 (22.4)**	**5 (8.9)**	**3.70***	**1.03-13.3**
	No	**194 (77.6)**	**5 (2.5)**		
TFI	**Yes**	**41 (16.4)**	**2 (4.9)**	**1.28**	**0.26-6.29**
	No	**209 (83.6)**	**8 (3.8)**		
Endometriosis	**Yes**	**22 (8.8)**	**2 (9.0)**	**2.75**	**0.54-13.83**
	No	**228 (91.2)**	**8 (3.5)**		

* Significant odds ratio

Table 6.5: Reproductive history and *C. trachomatis* prevalence (urine DNA) in infertile women.

Reproductive history		No. (%)	*C. trachomatis* infected No. (%)	Odds ratio	95% (CI)
Infertility	**Primary**	**182 (73.0)**	**10 (5.5)**	**1.91**	**0.40-8.98**
	Secondary	**68 (27.2)**	**2 (2.9)**		
Miscarriage	**Yes**	**41 (16.4)**	**2 (4.9)**	**1.02**	**0.21-4.83**
	No	**209 (83.6)**	**10 (4.8)**		
ART	**Yes**	**54 (21.6)**	**4 (7.4)**	**1.88**	**0.54-6.49**
	No	**196 (78.4)**	**8 (4.1)**		
Menstruation	**Regular**	**164 (65.6)**	**6 (3.6)**	**0.50**	**0.15-1.62**
	Irregular	**86 (34.4)**	**6 (6.9)**		
Infection	**Yes**	**137 (54.8)**	**8 (5.8)**	**1.69**	**0.49-5.76**
	No	**113 (45.2)**	**4 (3.5)**		
PCOS	**Yes**	**56 (22.4)**	**5 (8.9)**	**2.61**	**0.79-8.59**
	No	**194 (77.6)**	**7 (3.6)**		
TFI	**Yes**	**41 (16.4)**	**2 (4.9)**	**1.02**	**0.21-4.83**
	No	**209 (83.6)**	**10 (4.8)**		
Endometriosis	**Yes**	**22 (8.8)**	**0 (0.0)**	**0.40**	**0.02-7.16**
	No	**228 (91.2)**	**12 (5.3)**		

Table 6.6: Reproductive history and *C. trachomatis* prevalence (IgG) in infertile women.

Reproductive history		No. (%)	*C. trachomatis* infected IgG No. (%)	Odds ratio	95% (CI)
Infertility	**Primary**	**182 (73.0)**	**27 (14.8)**	**0.81**	**0.38-1.71**
	Secondary	**68 (27.2)**	**12 (17.6)**		
Miscarriage	**Yes**	**41 (16.4)**	**6 (14.6)**	**0.91**	**0.35-2.34**
	No	**209 (83.6)**	**33 (15.8)**		
ART	**Yes**	**54 (21.6)**	**12 (22.2)**	**1.78**	**0.83-3.82**
	No	**196 (78.4)**	**27 (13.7)**		
Menstruation	**Regular**	**164 (65.6)**	**28 (17.0)**	**1.40**	**0.66-2.97**
	Irregular	**86 (34.4)**	**11 (12.8)**		
Infection	**Yes**	**137 (54.8)**	**20 (14.6)**	**0.84**	**0.42-1.67**
	No	**113 (45.2)**	**19 (16.8)**		
PCOS	**Yes**	**56 (22.4)**	**15 (26.8)**	**2.59***	**1.24-5.37**
	No	**194 (77.6)**	**24 (12.4)**		
TFI	**Yes**	**41 (16.4)**	**6 (14.6)**	**0.91**	**0.36- 2.34**
	No	**209 (83.6)**	**33 (15.8)**		
Endometriosis	**Yes**	**22 (8.8)**	**6 (27.2)**	**2.21**	**0.80-6.07**
	No	**228 (91.2)**	**33 (14.5)**		

*Significant odds ratio

6.3.3 Follow up

6.3.3.1 Summary of diagnoses, treatment and outcome of n=250 infertile couples

Table 6.7 summarises diagnoses, treatment and treatment outcome of the 250 infertile couples recruited to the study. Briefly, the most common single reason for infertility was male factor infertility (40%) with PCOS (22.4%) tubal damage (16.4%) and unexplained (12.4%) being the next most common. Since couples could have more than one reason for their infertility, the list of diagnoses adds up to more than 100%.

With regard to the use of assisted conception, 123 couples received some kind of ART during the two year follow up including IVF (15.6%), ICSI (26.4%) and IUI (7.2%). Of those couples that underwent ART, 59 achieved a pregnancy and of those that did not 56 couples conceived pregnancy spontaneously. After 24 months, 25 couples were still undergoing treatment (data not shown).

6.3.3.2 Pregnancy outcome in *C. trachomatis* infected women

Table 6.8 shows (i) the number of women who achieved a pregnancy with or without infection either spontaneously or treatment assisted, and (ii) the pregnancy outcome respect with live birth or pregnancy loss in both ART and spontaneously conceived pregnancies. In summary, there were no statically differences with regard to achieving pregnancy or pregnancy outcome and *C. trachomatis* positivity, regardless of how this was defined (IgM, PCR, and IgG).

6.3.3.3 Pregnancy outcome in women with a *C. trachomatis* infected partner

Table 6.9 shows (i) the number of women who achieved pregnancy either spontaneously or treatment assisted who had partners with or without infection. The table also shows (ii) the pregnancy outcome respect with live birth or pregnancy loss in both ART and spontaneously conceived pregnancies. Briefly, there were no differences with regard to achieving pregnancy or pregnancy outcome in women whose partners were *C. trachomatis* positive regardless of how this was defined (IgM, PCR, and IgG).

Table 6.7: Diagnoses, treatment and outcome summary of the infertile women (n=250) after 24 months follow up.

Diagnoses	No of couples.	Percentage (%)
Principal diagnoses:		
Male factor	**100**	**40.0**
PCOS	**56**	**22.4**
Tubal damage (adhesions)	**41**	**16.4**
Unexplained	**31**	**12.4**
Oligomenorrhea	**30**	**12.0**
Endometriosis	**22**	**8.8**
Treatments:		
None	**64**	**25.6**
Ovulation induction	**63**	**25.2**
ICSI	**66**	**26.4**
IVF	**39**	**15.6**
IUI	**18**	**7.2**
Achieved pregnancy:	**115**	**46.0**
Spontaneously	**56**	**48.7**
Treatment related	**59**	**51.3**

Table 6.8: *Chlamydia trachomatis* antibodies (IgM & IgG) and PCR of urine DNA in the female partner showing: (i) probability of achieving pregnancy and (ii) pregnancy outcome.

(i)		Achieved pregnancy		Odds Ratio (95%CI)	
		Spontaneous	ART	Spontaneous	ART
IgM	Pos	2/10	4/10	0.88 (0.19-4.15)	1.80 (0.54-5.98)
	Neg	54/240	55/240		
DNA	Pos	4/12	1/12	1.56 (0.48-5.08)	0.33 (0.04-2.59)
	Neg	52/238	58/238		
IgG	Pos	11/39	13/39	1.22 (0.56-2.67)	1.92 (0.96-4.09)
	Neg	45/211	46/211		

(ii)		Pregnancy outcome				Odds Ratio (95%CI)			
		Spontaneous		ART		Spontaneous		ART	
		Live birth	Pregnancy loss	Live birth	Pregnancy loss	Live birth	Pregnancy loss	Live birth	Pregnancy loss
IgM	Pos	2/10	0/10	3/10	1/10	1.00 (0.21-4.71)	2.09 (0.10-40.48)	1.64 (0.63-6.22)	2.55 (0.29-22.17)
	Neg	48/240	6/240	45/240	10/240				
DNA	Pos	4/12	0/12	1/12	0/12	1.72 (0.53-5.68)	2.09 (0.10-41.41)	0.34 (0.04-3.02)	1.25 (0.06-23.12)
	Neg	47/238	5/238	50/238	8/238				
IgG	Pos	9/39	2/39	11/39	2/39	1.24 (0.54-2.80)	4.41(0.60-32.28)	1.79 (0.82-3.92)	1.47 (0.28-7.56)
	Neg	41/211	4/211	38/211	8/211				

Table 6.9: *Chlamydia trachomatis* antibodies (IgM & IgG) and PCR of urine DNA in the male partner and: (i) probability of achieving pregnancy and (ii) pregnancy outcome.

(i)		Achieved pregnancy		Odds Ratio (95%CI)	
		Spontaneous	ART	Spontaneous	ART
IgM	Pos	0/3	1/3	0.73 (0.04-14.83)	1.42 (0.15-13.97)
	Neg	56/247	58/247		
DNA	Pos	4/11	2/11	1.72 (0.52-5.65)	0.75 (0.16-3.49)
	Neg	52/239	57/239		
IgG	Pos	14/45	12/45	1.77 (0.86-3.65)	1.21 (0.58-2.54)
	Neg	42/205	47/205		

(ii)		Pregnancy outcome				Odds Ratio (95%CI)			
		Spontaneous		ART		Spontaneous		ART	
		Live birth	Pregnancy loss	Live birth	Pregnancy loss	Live birth	Pregnancy loss	Live birth	Pregnancy loss
IgM	Pos	0/3	0/3	1/3	0/3	0.83 (0.04-17.00)	6.25 (0.28-138.0)	1.78 (0.17-17.37)	3.87 (0.18-82.34)
	Neg	49/247	7/247	47/247	11/247				
DNA	Pos	4/11	0/11	2/11	0/11	1.97 (0.60-6.49)	1.84 (0.09-36.27)	0.94 (0.20-4.39)	0.98 (0.05-17.95)
	Neg	46/239	6/239	46/239	11/239				
IgG	Pos	14/45	0/45	11/45	1/45	1.55 (0.76-3.16)	0.32 (0.02-5.95)	1.05 (0.69-3.27)	0.39 (0.05-3.20)
	Neg	36/205	6/205	37/205	10/205				

6.3.4 Risk of TFI and *C. trachomatis* positivity in both partners

Table 6.10 shows the relationship between *C. trachomatis* infection in both infertile females and males, with the diagnosis of tubal factor infertility in the female partner. In summary, this shows that TFI, in the female partner was unrelated to the status of *C. trachomatis* in either herself or her partner, as defined by PCR or serology (IgM & IgG).

Table 6.10: *Chlamydia trachomatis* antibodies and urine DNA in the female and male partner and chances of TFI.

***C. trachomatis* Status in female**		**TFI**	**Odds Ratio (95%CI)**	***C. trachomatis* Status in male**		**Partner's TFI**	**Odds Ratio (95%CI)**
IgM	**Positive**	**2/10**	**1.24(0.26-5.92)**	**IgM**	**Positive**	**0/3**	**1.93(0.04-13.59)**
	Negative	**39/240**			**Negative**	**41/247**	
DNA	**Positive**	**0/12**	**0.20(0.01-3.61)**	**DNA**	**Positive**	**2/11**	**1.14(0.23-5.47)**
	Negative	**41/238**			**Negative**	**39/239**	
IgG	**Positive**	**6/39**	**1.22(0.46-3.19)**	**IgG**	**Positive**	**9/45**	**1.35(0.60-3.14)**
	Negative	**35/211**			**Negative**	**32/205**	

6.4 Discussion

The main findings of this chapter were that the history of urethritis and working as a driver in the men and PCOS in women were statistically related to *C. trachomatis* infection. However, *C. trachomatis* infection was not related either to TFI or the chances of achieving pregnancy, experiencing pregnancy loss, or the chances of a live birth.

To examine the first of these findings, it is perhaps no surprise that a history *C. trachomatis* infection by urine DNA was significantly associated with the history of urethritis in the male partners (OR= 34.82; 95%CI= 8.45-143.40). This observation is similar to other studies (Holms *et al*., 1975; Schachter, 1999; Gonzales *et al*., 2004; Taylor and Haggerty, 2011) and confirms the infection of the genitourinary tract is the most common clinical appearances in men following *C. trachomatis* infection. The presence of *C. trachomatis* DNA in urine samples indicates a current infection. The reason that urethritis was not observed in antibody positive men, could be that in individuals with a normal immune response, it is usual that a high infectious dose of *C. trachomatis* would result in an antibody response of IgM after two weeks and IgG a few weeks later. In an infection with a low dose of *C. trachomatis* it is still possible to detect DNA because of the high sensitivity of the test. However as the antibody test is not as sensitive as the DNA test it may not be possible to detect antibodies in these cases.

Interestingly the male partners who are drivers, were more likely to have exposure to *C. trachomatis* (OR=2.37; 95%CI= 1.03-5.45) as defined by IgG positivity. This is in agreement with the findings of Leung *et al*., (2009) that investigated *C. trachomatis* prevalence between cross-border truck drivers in Hong Kong. The prevalence was not extremely high, but it was suggested that such drivers could have been more promiscuous and engaged in sex with commercial sex worker and extramarital sex partners therefore increasing the chances of infection.

In female partners, the results showed *C. trachomatis* positive serology (IgM & IgG) was associated with PCOS (IgM (+): OR= 3.70; 95% CI= 1.03-13.3; IgG (+): OR= 2.59; 95%CI= 1.24-5.37). This is similar to the finding of Morin-Papunen *et al*., (2010) who investigated the correlation of systemic inflammation induced by chlamydia infection and symptoms of PCOS. PCOS is the most common cause of hyperandrogenic

anovulatory infertility and is also associated with hyperinsulinemia due to resistance to insulin (metabolic disorder). They found the chronic inflammation because of *C. trachomatis* could contribute in the metabolic and hormonal disorder of PCOS (Morin-Papunen *et al*., 2010) as chlamydial infection can be cause of insulin resistance and systemic inflammation (Fernandez-real *et al*., 2006).

In the present study the most common reason for primary and secondary infertility was male factor (40.0%). This is as expected, since in most other studies male infertility is the most common reason for a couple's infertility (Farhi and Ben-Haroush, 2011; Eisenberg *et al*., 2012). The second and third most common reason for infertility were for PCOS and tubal damage and this is also as expected (Farhi and Ben-Haroush, 2011; Eisenberg *et al*., 2012). This suggests that the infertile couple enrolled to the study are representative of those recruited to other studies. It also suggests that the aetiology of infertility in Iran is similar to other countries.

In the present study, IgG positivity was not associated with TFI in women, when either women or their partners were positive (see Table 6.10). This is in contrast to Idahl and colleagues who found a relationship between of IgG positive and TFI where women but not their partners were serology positive (Idahl *et al*., 2004). Different studies have shown the relationship of *C. trachomatis* antibodies and TFI (Dabekausen *et al*., 1994; Eggert-Kruse *et al*., 1997; Paavonen and Eggert-Kruse, 1999, Akande *et al*., 2003, Mardh, 2004; Coppus *et al*., 2007, El Akande *et al*., 2010; Hakim *et al*., 2010). Apart from different diagnostic methods among these studies (Dabekausen *et al*., 1994; El Hakim *et al*. 2010), they also investigated the efficiency of doing laparoscopy based on the positive result for chlamydia and medical history (Coppus *et al*., 2007; El Hakim *et al*. 2010), or they investigated tubal damage with special regard to chlamydial salpingitis (Mardh, 2004). In this study, women were not symptomatic and the serology results were obtained after patient recruitment and the completion of all diagnostic procedures. Therefore is arguably better than other studies by recruiting couples blindly without reference to their diagnosis or reason for infertility. Among 41 women with a TFI diagnosis, only 6 female and 9 male partners were IgG positive, with only one couple where both were IgG positive. The rest (26 women) were negative for IgG antibody to chlamydia. MIF staining is the gold standard of *C. trachomatis* detection, (see section 2.3.1). The low number of positive results from this method might be the

most important cause of low concordance within couples. The method used in this study did not measure the different titre and level of antibody that could be considered positive with other studies. According to the kit instructions the dilution rate for IgG serum antibody was only 1:64 and did not include different dilution rates. Although this is weakness of our study, the total number of IgG positives (n=39 women) is comparable with other previous studies (Idahl *et al*., 2004; 2007). Therefore, the present study draws attention to the interesting finding that antibody IgG to chlamydia was not related to TFI and this could be genuine finding or due to a high number of negative results that can effect on the odds ratio.

Other infection (e.g. TB) and multiple reasons including ovarian endometriosis, the history of abortion and curettage, history of using intra-uterine contraceptive device (IUD) must also be considered as a possible aetiology of tubal damage. Although, these reasons might be the cause of tubal damage or a masking factor (antibiotic treatment in the early stage of lower genital tract infection) of the IgG results in any study, in Iran genital tuberculosis (TB) is specifically common cause of infertility (Namvar Jahromi *et al*., 2001). The incidence (per 100,000 people) of TB in Iran (17), UK (13), Sweden (6.8), Germany (4.8) and USA (4.1) was last reported in 2010; according to a World Bank report (WHO, 2012).

Of 250 infertile couples, one hundred and fifteen got pregnant either spontaneously (48.7%) or by use of ART (51.3%). Idahl *et al*., (2004), who followed up 244 infertile couples for a mean of 37 (range 14-54 months), found that pregnancy was achieved spontaneously in 58.7% of couples and 41.3% following treatment. The higher spontaneous pregnancy rate may be explained by Idahl's longer follow-up time (14-54 months) but this study's increase in ART success might be because more couples received treatment. In this study 25.6% had not received treatment at the end of follow-up whereas in Idahl's it was 48.3%. Also in this study, the male factor was most common reason and this could be treated by ICSI, but in Idahl's study unexplained infertility was most common reason.

To see how *C. trachomatis* infection was related to pregnancy rate, the results for serology and PCR in both partners were considered against the chances of achieving pregnancy and pregnancy outcome (including pregnancy loss). Interestingly, the infection was not associated with chances of achieving pregnancy and live birth or

pregnancy loss when either of the female or male partners were positive for *C. trachomatis* (by any method). The results were in contrast with Idahl *et al*., (2004) who found that IgG positivity in the male partner was associated with not achieving pregnancy in their partner, whereas the positivity in female partners was not associated with achieving pregnancy (Idahl *et al*., 2004).

In conclusion, this study provides little evidence for the role of *C. trachomatis* in the fertility of couples and the incidence of TFI in women referred for infertility. In the next and final chapter of this thesis the results across all chapters will be considered and discussed in context with wider the literature.

7 Chapter 7: General discussion

This thesis set out to evaluate the role of *C. trachomatis* and *M. genitalium* in infertile couples in a region of Iran. No previous work has been reported in this area in Iran and the study evaluated the prevalence and the relationship of these infections with semen quality, semen inflammatory markers and sperm DNA measurements, also the effect of *C. trachomatis* infection on pregnancy rate and pregnancy outcome.

7.1 Summary of findings

The starting point of this work was a population-based prevalence study of infertile couples and fertile women using PCR and serology. The main strength of this study is the information about prevalence of *C. trachomatis* & *M. genitalium* among asymptomatic population who were not seeking treatment. Moreover, a second strength was the PCR based diagnoses on urine samples of all infertile couples compared to Idahl *et al*., (2004) who performed PCR only on IgG positive patients. Finally, this study also achieved an excellent follow-up rate of 95%. However our cohort was small to investigate the impact of *C. trachomatis* infection and the relatively short follow-up period (24 months) may be two weaknesses of the study.

The study in Chapter 3 showed low prevalence of *C. trachomatis* infection as determined by NPPCR and serology, and a zero incidence of *M. genitalium* as defined by PCR. Previous studies show variable rates according to the population tested and the methods used. The prevalence of *C. trachomatis* found in this thesis was low but comparable to other studies (Eggert-Kruse *et al*., 1997; 2003; Hosseinzadeh *et al*., 2004; Idahl *et al*., 2004; Hamdad-Daoudi *et al*., 2004). In addition to low prevalence of *C. trachomatis*, the results also showed a low concordance within couples. The most likely reason for this is the low number of patients with positive serology or PCR tests and this was discussed in section 3.4. Moreover, the PCR results from urine samples might have lower positivity than the actual incidence because urine samples do not include many cells and the presence of PCR inhibitor and also the kit which used (see section 3.4). The results of the study supported previous studies suggesting that FVU is a more practical specimen in asymptomatic men to detect *C. trachomatis* (Chernesky *et al*., 1994; Michel *et al*., 2007; Wiggins *et al*., 2009) as no positive was observed in semen samples of men that had past exposure to *C. trachomatis* determined by serology.

As anticipated, the results in Chapter 3 showed an increased prevalence of *C. trachomatis* IgG antibody in the infertile population compared to women with proven fertility that was not statistically significant. No *M. genitalium* was observed in urine and semen samples. As discussed in Chapter 3 the reason might be the inappropriate kit and nature of samples. That also might be caused of selection bias as poorer people (who are reported to have higher prevalence of *M. genitalium*) cannot afford treatment in Iran, as people should pay in public medical centre and that can affect the prevalence of this organism (Yeganeh *et al*., 2013). To achieve cost-effectiveness in Iran further multi- central studies will be needed on younger infertile couples.

In the fourth chapter, this thesis evaluated the relationship between *C. trachomatis* infection and semen parameters and seminal inflammatory markers. The main strength of this study, compared to previous studies, was that it evaluated serology (IgA, IgM, and IgG) and PCR (semen & urine DNA) both to detect past and current infection. The conclusion was that the only semen parameter affected by *C. trchomatis* infection as defined by PCR, was semen volume which is in contrast with Hosseinzadeh *et al*., (2004) but in support Kokab *et al*., (2010). However mean semen pH was significantly higher in IgG positive men in support of Marconi *et al*., (2009) and Kokab *et al*., (2010). The results for seminal inflammatory markers were comparable with other previous work (Eggert-Kruse *et al*., 2001; Kokab *et al*., 2010) and high IL-6 and IL-8 levels suggested that these cytokines may be a marker of upper genital tract infection especially prostatitis (Al-Mosley and Eley, 2007). As *C. trachomatis* is treatable, it could be diagnosed by screening infertile couples earlier to detect this infection. Taylor *et al*., (2005) found a link between STD infections including chlamydia and prostate cancer and Nelson *et al*., (2003) showed link between prostatic inflammation and prostate cancer. However there is controversial idea about the infection sequelae such as BPH and cancer of the prostate among experts.

In Chapter five, sperm DNA measurements were studied in infected men as defined by serology and PCR. The TUNEL assay was performed on semen samples from all 250 males recruited and additional measures of sperm chromatin were undertaken on a sub-population of 80 men. The percentage of DNA fragmentation using the TUNEL assay showed 12.75±8.36 (mean±SD) overall among infertile men. As DNA damage can be underestimated by the TUNEL assay (Mitchell *et al*., 2011), the results may not have

showed the true percentage of fragmentation. The study weakness is that the results were obtained by microscopic examination of stained smears, whereas other researchers (Marchetti *et al*., 2002; Shen *et al*., 2002; Gillan *et al*., 2005; Mitchell *et al*., 2011) have used flow cytometry to examine a greater number of cells. Flow cytometry is more statistically reliable as it analyses a high number of cells and therefore is considered more accurate and more discriminative. Recently, studies on DNA integrity that focuses on the genomic integrity of the male gamete has been further increased because of the growing concern about transmission of genetic disease through ICSI (Barroso *et al*., 2000). However, there was no evidence in the patients recruited to this study that they had infertility because of high levels of DNA damage in sperm. There is controversy about the role sperm DNA testing has in the infertility work up and this is because there is no strong evidence (Barratt *et al*., 2010; ASRM, 2013) that sperm DNA abnormality results in ART failure or pregnancy outcome. Couples with long term unexplained infertility are more likely to gain more advantage of using these tests (Sakkas & Alvarez, 2010). On the other hand, it has been suggested that sperm DNA damage evaluation may be better than semen analysis (Zini *et al*., 2001) for prediction of outcome of spontaneous pregnancy and assisted reproductive techniques. Also DNA fragmentation levels more than 30% are considered as a cut off level that spontaneous pregnancy is not achievable and is linked to ART failure (Zini *et al*., 2001; Evenson *et al*., 1991, 2002; Borini *et al*., 2006; Nallella *et al*., 2006).

The results from chromatin assessment (in a sub-population of 80 men) showed higher percentage damage (AB: 29.16±14.75; AO: 38.24±11.77 and CMA3: 38.11±9.17) compare with TUNEL results. Another study weakness was that the semen sample on which sperm DNA measurements were made, was not the same as the one used in the ART cycle or the ejaculate which lead to any spontaneous pregnancy. However, we know that DNA damage can highly vary in multiple samples and is influenced by environmental or lifestyle factors as well (Pacey, 2010) and therefore the measures reported here may not be representative.

In Chapter six, the thesis investigated the relationship between past reproductive and medical history and *C. trachomatis* infection. In the male partner, as anticipated, a history of urethritis was associated with *C. trachomatis* DNA in urine and the infection was more likely to be present in men who worked as drivers. In the female partners, *C.*

trachomatis positive serology was associated with PCOS as observed by Morin-Papunen *et al*., (2010). In contrast with other studies (Sellors *et al*., 1988; Dabekausen *et al*., 1994; Mardh, 2004) this study did not support the relationship between *C. trachomatis* and TFI. This may be because previous studies have investigated *C. trachomatis* using culture among women that showed TFI, by taking samples of uterine tube or cervix by biopsy or swab. However, other studies (Dabekausen *et al*., 1994; Eggert-Kruse *et al*., 1997) used serology to determine chlamydial infection in women with abnormal HSG results. Given that the sperm can be stored in uterine tubes (Suarez & Pacey, 2006) and *C. trachomatis* is able to attach to sperm (Hosseinzadeh *et al*., 2000) then it was perhaps not surprising that TFI was not related to these organisms since in the population of men recruited to this study, neither *C. trachomatis* or *M. genitalium* were detected in semen (see section 3.3.2 & 3.3.4). Also the women who were infected may only have had lower genital tract infection that had either been treated or had been cleared by their immune system and therefore this could explain why that *C. trachomatis* infection was not related to either pregnancy rate (assisted or unassisted) or pregnancy outcome in this population.

7.2 Implications of the thesis findings

In the following sections, the thesis will conclude with a wider discussion about the topic with some suggestions for further future work.

7.2.1 Is *C. trachomatis* a problem?

When chlamydia is diagnosed, women will understandably be concerned about their future fertility. In the Western countries the relationship between *C. trachomatis* and future fertility is often reported in the media or through National Health Service (NHS) campaigns and women are generally informed about the consequence of STDs. However this is not the same in Iran where the media does not report STDs and the general population are relatively uninformed about any relationship between chlamydia and infertility. Since the findings of this thesis showed this infection is not related to infertility, it must be questioned whether *C. trachomatis* infection really causes infertility?

There is a significant body of literature showing the relationship between PID, TFI and ascending chlamydia infection (discussed in Chapter 6), however, this thesis supports

previous studies (Mardh, 2004; Geisler *et al*., 2008) which show that even in unrecognized infection, there is no evidence of serious complication and sequelae (Mardh, 2004) without upper genital tract infection and reinfection. Also spontaneous clearance occurs in 30-50% of lower genital tract infection (Geisler *et al*., 2008) and only persistent infections develop to complications. In addition, in adverse pregnancy outcome the host immunological response is more important than micro-organisms (Land *et al*., 2010) and also previous work is based on retrospective studies as prospective studies are unethical to conduct (Wallace *et al*., 2008; Land *et al*., 2010). For example, in the systematic review by Wallace *et al*., (2008) of 3,349 studies evaluated only one study met the inclusion criteria and this was found to have methodological flaws. Therefore, Wallace *et al*., (2008) concluded that the current assumptions of prevalence and relationship of *C. trachomatis* and tubal damage were incorrect with a poor evidence base. Moreover, the role of epididymitis as a result of developing lower genital tract infection is not well understood in male infertility (Karinen, 2005). *C. trachomatis* infection among the working class, in stable relationships, infertile population of this study was not a cause of their infertility. However, it should be recognized that this conclusion might be different in other populations and locations. Further future studies will be needed to make clear the magnitude of *C. trachomatis* infection in infertility.

7.2.2 Why we are screening?

The primary aim of screening is reduce morbidity by the early detection of infection and the secondary aim is to decrease of overall prevalence of infection. Opportunistic and proactive screening programmes in Western countries screen women < 25 years when they are sexually active (SIGN, 2009, Low *et al*., 2011). As this study did not show association between chlamydia and infertility, this thesis would seem to undermine the very logic of such screening programmes.

It has been suggested by previous literature that current screening programme are not based on strong evidence (Katz *et al*., 1994; van Valkengoed *et al*., 2004; Low *et al*., 2006, 2007; Wallace *et al*., 2008; Land *et al*., 2010). For example, the assertion that *C. trachomatis* screening reduced morbidity in Sweden is not supported by data from other countries (Low *et al*., 2007). A recent cross-sectional survey in Europe stated that using an appropriate diagnostic method is essential for effective screening (Low *et al*., 2011)

and this is more likely to be achieved by using national guidelines and regular audit. Therefore if the aim of screening is reduce morbidity related to fertility issues; it should consider selected population and couple screening rather than only women as our results showed poor concordance within couples. In Iran, there are no screening programmes based on national guidelines or regular audit for the prevalence of STDs, but our result showed the prevalence of *C. trachomatis* is comparable with other countries. Considering that our results have not found any relationship with chlamydia infection and its consequences such as PID, EP and infertility, this may suggest screening programmes are only needed in Iran for early detection of disease and prevent of spread disease in sexual active people (< 25 years) or in pregnant women to prevent of neonatal infection as this infection could be persistent.

7.2.3 Is screening cost-effective?

To evaluate cost-effectiveness of screening, selective screening is strongly recommended (Mylonas, 2012). Since prevalence data is not reliable, estimation of screening cost is problematic and also cost of clinical approaches to infertility are less than screening (Hu *et al*., 2004). This is because the model that is used for cost-effective ness is based on an overestimate assumption of complications (van Valkengoed *et al*., 2004; Low *et al*., 2007) and there is no evidence of serious complications (Wallace *et al*., 2008, Garnett, 2008; Low *et al*., 2007). Therefore this is an expensive intervention under assumption. The reason for high rates assumption is that prospective studies are unethical and most predictions for cost-saving come from the costs of treatment and complications prevented (van Valkengoed *et al*., 2001). Moreover, most studies were performed in high risk populations and these are at greater risk of complications (van Valkengoed *et al*., 2004). As high risk of complications have only been observed with repeated infection and are less than expected in the general population, it has been argued that the benefit of screening programmes have been exaggerated (Low *et al*., 2006).

For example, screening in Western countries seems expensive. Estimation of screening cost per quality-adjusted life-years (QALY) gained every 2 years in Netherlands would cost €20,000 (de Vries *et al*., 2008), and annual QALY gained in the UK would cost £27,000 (Adams *et al*., 2007). In both of these studies men as well as women were included. Chlamydia screening programme in Ireland was found to be expensive

(€94,717 per QALY gained) and is unlikely to be considered by policy makers (Gillespie *et al*., 2012). Researchers in the USA found the cost of screening only young women was US$25,000 per QALY gained annually (Hu *et al*., 2004). In fact all of these reports were performed based on the overestimate of complications like PID, TFI and EP. The calculation based on observed occurrence of complications in Amsterdam showed very low rates of complications (van Valkengoed *et al*., 2004) compared to calculation based assumptions. Considering the lack of diagnostic quality in the diagnosis of PID and high burden impact of its diagnoses and treatment on the NHS (and also for IVF treatment), it has been suggested that it would be more cost effective to spend money on diagnosis and treatments rather than screening (Simms *et al*., 2000). In low prevalence population some people believed chlamydia testing is "wasting money" (BBC News/Health, 2009).

Few studies have shown cost-effectiveness of screening and treatment (Land *et al*., 2010). To improve the evaluation of screening activities, it has been suggested that future research should consider the risk of reinfection, persistent and PID after *C. trachomatis* infection (Hu *et al*., 2006). Proactive screening was suggested to selected population with high prevalence of infection that might be cost effective and cost-saving (Fenton & Ward, 2004; Land *et al*., 2010; Mylonas, 2012).

7.2.4 Future work

The findings of this thesis suggested that *C. trachomatis* is not a problem in infertile couples who were recruited from the Yazd IVF centre in Iran. However there is no data about the effect of long term persistent infection and horizontal spreading neonatal infections that may have a large health burden impact.

There are no national guidelines available for chlamydia screening in Iran in the same way that there are in Western countries such as the UK. Therefore to convince the Iranian Ministry of Health to consider developing screening guidelines and funding screening strategies, stronger and larger studies will be needed to evaluate the benefits and disadvantage of screening programme including side effect of screening or treatment of long term complications. This study only illustrates the prevalence and effect of *C. trachomatis* among infertile couples from the working or lower classes, who were in stable relationships in central and South of Iran. It is suggested that future multi-

centre studies of young couples are needed to find more accurate prevalence data using PCR on FVU samples as the specimen of choice in both males and females. It would be necessary to evaluate the cost of treating complications (i.e., infertility) and screening cost in patients over a long term follow up to optimize better screening strategy. As Western countries spend a huge amount of money (section 7.2.3) and also people involved with their screening programme, study like the present study might be a good start to change and review their guidelines.

On the other hand, this thesis did not show *M. genitalium* infection among infertile couples. Further future studies will be needed in different level of people without any selection bias to find robust prevalence data.

7.2.5 Conclusions

The findings of this thesis indicate in the study population, *C. trachomatis* infection was not related to either female or male infertility. The results suggest a comparable incidence of this infection with other studies; however, the present study does not support opportunistic screening for early detection to reduce morbidity of complications because there were no complications related to this infection in the women recruited. This study was the first of its kind in Iran and it will be need to be repeated in other Iranian cities to find the probability of any complication related to infection. The role of *C. trachomatis* and/or *M. genitalium* as infectious agent is still controversial and long term follow up studies will be needed to clarify their significance.

8 References

Abd-Raboh S, Saleh H A, Hanafi N F & Darwish H B. (2011) The detection of *Chlamydia Trachomatis* Antigen in cervical secretions and serum antibodies in infertile females undergoing ICSI and its impact on pregnancy success. *Life Sci J* 8, 259-263.

Adams E J, Turner K M E & Edmunds W J. (2007) The cost effectiveness of opportunistic chlamydia screening in England. *Sex Transm Infect* 83.

Agarwal A & Said T M. (2003) Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 9, 331-345.

Agrawal T, Vats V, Salhan S & Mittal A. (2007) Mucosal and peripheral immune responses to chlamydial heat shock proteins in women infected with *Chlamydia trachomatis*. *Clin Exp Immunol* 148, 461-468.

Aitken R J. (1994) A free radical theory of male infertility. *Reprod Fertil Dev* 6, 19-23; discussion 23-14.

Aitken R J, Baker M A & Sawyer D. (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* 7, 65-70.

Akande V, Turner C, Horner P, Horne A & Pacey A. (2010) Impact of *Chlamydia trachomatis* in the reproductive setting: British Fertility Society Guidelines for practice. *Hum Fertil (Camb)* 13, 115-125.

Akande V A, Hunt L P, Cahill D J, Caul E O, Ford W C & Jenkins J M. (2003) Tubal damage in infertile women: prediction using chlamydia serology. *Hum Reprod* 18, 1841-1847.

Al-Mously N, Cross N A, Eley A & Pacey A A. (2009) Real-time polymerase chain reaction shows that density centrifugation does not always remove *Chlamydia trachomatis* from human semen. *Fertil Steril* 92, 1606-1615.

Al-Mously N & Eley A. (2007) Interaction of *Chlamydia trachomatis* serovar E with male genital tract epithelium results in secretion of proinflammatory cytokines. *J Med Microbiol* 56, 1025-1032.

Alexander S, Martin I & Ison C. (2007) Confirming the *Chlamydia trachomatis* status of referred rectal specimens. *Sex Transm Infect* 83, 327-329.

Alvarez J G, Sharma R K, Ollero M, Saleh R A, Lopez M C, Thomas A J, Jr., Evenson D P & Agarwal A. (2002) Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. *Fertil Steril* 78, 319-329.

Anagrius C, Lore B & Jensen J S. (2005) *Mycoplasma genitalium*: prevalence, clinical significance, and transmission. *Sex Transm Infect* 81, 458-462.

Andrade-Rocha F T. (2003) Ureaplasma urealyticum and Mycoplasma hominis in men attending for routine semen analysis. Prevalence, incidence by age and clinical settings, influence on sperm characteristics, relationship with the leukocyte count and clinical value. *Urol Int* 71, 377-381.

Awwad Z M, Al-Amarat A A & Shehabi A A. (2003) Prevalence of genital chlamydial infection in symptomatic and asymptomatic Jordanian patients. *Int J Infect Dis* 7, 206-209.

Baczynska A, Fedder J, Schougaard H & Christiansen G. (2007) Prevalence of mycoplasmas in the semen and vaginal swabs of Danish stallions and mares. *Vet Microbiol* 121, 138-143.

Bakhtiari A & Firoozjahi A. (2007) *Chlamydia trachomatis* infection in women attending health centres in Babol: prevalence and risk factors. *East Mediterr Health J* 13, 1124-1131.

Bakken I J & Ghaderi S. (2009) Incidence of pelvic inflammatory disease in a large cohort of women tested for *Chlamydia trachomatis*: a historical follow-up study. *BMC Infect Dis* 9, 130.

Barratt C L, Aitken R J, Bjorndahl L, Carrell D T, de Boer P, Kvist U, Lewis S E, Perreault S D, Perry M J, Ramos L, Robaire B, Ward S & Zini A. (2010) Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications--a position report. *Hum Reprod* 25, 824-838.

Barratt C L, Robinson A, Spencer R C, Kinghorn G R, White A, Harrison P E, Kessopoulou E & Cooke I D. (1990) Seminal peroxidase positive cells are not an adequate indicator of asymptomatic urethral genital infection. *Int J Androl* 13, 361-368.

Barroso G, Morshedi M & Oehninger S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338-1344.

Baseman J B, Cagle M, Korte J E, Herrera C, Rasmussen W G, Baseman J G, Shain R & Piper J M. (2004) Diagnostic assessment of *Mycoplasma genitalium* in culture-positive women. *J Clin Microbiol* 42, 203-211.

Baseman J B, Reddy S P & Dallo S F. (1996) Interplay between mycoplasma surface proteins, airway cells, and the protean manifestations of mycoplasma-mediated human infections. *Am J Respir Crit Care Med* 154, S137-144.

Batteiger B E. (1996) The major outer membrane protein of a single *Chlamydia trachomatis* serovar can possess more than one serovar-specific epitope. *Infect Immun* 64, 542-547.

Batteiger B E & Rank R G. (1987) Analysis of the humoral immune response to chlamydial genital infection in guinea pigs. *Infect Immun* 55, 1767-1773.

Bax C J, Mutsaers J A, Jansen C L, Trimbos J B, Dorr P J & Oostvogel P M. (2003) Comparison of serological assays for detection of *Chlamydia trachomatis* antibodies in different groups of obstetrical and gynecological patients. *Clin Diagn Lab Immunol* 10, 174-176.

Beagley K W & Timms P. (2000) *Chlamydia trachomatis* infection: incidence, health costs and prospects for vaccine development. *J Reprod Immunol* 48, 47-68.

Beatty W L, Byrne G I & Morrison R P. (1993) Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. *Proc Natl Acad Sci U S A* 90, 3998-4002.

Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B & Guerin J F. (2007) Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertil Steril* 87, 93-100.

Berry S J, Coffey D S, Walsh P C & Ewing L L. (1984) The development of human benign prostatic hyperplasia with age. *J Urol* 132, 474-479.

Bezold G, Politch J A, Kiviat N B, Kuypers J M, Wolff H & Anderson D J. (2007) Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia. *Fertil Steril* 87, 1087-1097.

Black C M. (1997) Current methods of laboratory diagnosis of *Chlamydia trachomatis* infections. *Clin Microbiol Rev* 10, 160-184.

Bollmann R, Engel S, Petzoldt R & Gobel U B. (2001) *Chlamydia trachomatis* in andrologic patients--direct and indirect detection. *Infection* 29, 113-118.

Bornman M S, Ramuthaga T N, Mahomed M F, Greeff A S, Crewe-Brown H H & Reif S. (1998) Chlamydial infection in asymptomatic infertile men attending an andrology clinic. *Arch Androl* 41, 203-208.

Brunham R C, Binns B, Guijon F, Danforth D, Kosseim M L, Rand F, McDowell J & Rayner E. (1988) Etiology and outcome of acute pelvic inflammatory disease. *J Infect Dis* 158, 510-517.

Budai I. (2007) *Chlamydia trachomatis*: milestones in clinical and microbiological diagnostics in the last hundred years: a review. *Acta Microbiol Immunol Hung* 54, 5-22.

Cai T, Wagenlehner F M, Mazzoli S, Meacci F, Mondaini N, Nesi G, Tiscione D, Malossini G & Bartoletti R. (2012) Semen quality in patients with *Chlamydia trachomatis* genital infection treated concurrently with prulifloxacin and a phytotherapeutic agent. *J Androl* 33, 615-623.

Carder C, Mercey D & Benn P. (2006) *Chlamydia trachomatis*. *Sex Transm Infect* 82 Suppl 4, iv10-12.

Casin I, Vexiau-Robert D, De La Salmoniere P, Eche A, Grandry B & Janier M. (2002) High prevalence of *Mycoplasma genitalium* in the lower genitourinary tract of women attending a sexually transmitted disease clinic in Paris, France. *Sex Transm Dis* 29, 353-359.

Castro P, Xia C, Gomez L, Lamb D J & Ittmann M. (2004) Interleukin-8 expression is increased in senescent prostatic epithelial cells and promotes the development of benign prostatic hyperplasia. *Prostate* 60, 153-159.

Cates W, Jr. (1996) Contraceptive choice, sexually transmitted diseases, HIV infection, and future fecundity. *J Br Fer Soc* 1, 18-22.

Cates W, Jr. & Wasserheit J N. (1991) Genital chlamydial infections: epidemiology and reproductive sequelae. *Am J Obstet Gynecol* 164, 1771-1781.

Cengiz T, Aydoganli L, Baykam M, Mungan N A, Tuncbilek E, Dincer M, Yakupoglu K & Akalin Z. (1997) Chlamydial infections and male infertility. *Int Urol Nephrol* 29, 687-693.

Cevenini R, Sarov I, Rumpianesi F, Donati M, Melega C, Varotti C & La Placa M. (1984) Serum specific IgA antibody to *Chlamydia trachomatis* in patients with chlamydial infections detected by ELISA and an immunofluorescence test. *J Clin Pathol* 37, 686-691.

Chamani-Tabriz L, Tehrani M J, Akhondi M M, Mosavi-Jarrahi A, Zeraati H, Ghasemi J, Asgari S, Kokab A & Eley A R. (2007) *Chlamydia trachomatis* prevalence in Iranian women attending obstetrics and gynaecology clinics. *Pak J Biol Sci* 10, 4490-4494.

Chan E L, Brandt K, Kozoriz D, Mushanski L, Spence C & Horsman G B. (1994) The use of a confirmatory assay to increase the sensitivity and specificity of the Chlamydiazyme test. *Am J Clin Pathol* 102, 724-728.

Chow J M, Yonekura M L, Richwald G A, Greenland S, Sweet R L & Schachter J. (1990) The association between *Chlamydia trachomatis* and ectopic pregnancy. A matched-pair, case-control study. *Jama* 263, 3164-3167.

Claas H C, Melchers W J, de Bruijn I H, de Graaf M, van Dijk W C, Lindeman J & Quint W G. (1990) Detection of *Chlamydia trachomatis* in clinical specimens by the polymerase chain reaction. *Eur J Clin Microbiol Infect Dis* 9, 864-868.

Clausen H F, Fedder J, Drasbek M, Nielsen P K, Toft B, Ingerslev H J, Birkelund S & Christiansen G. (2001) Serological investigation of *Mycoplasma genitalium* in infertile women. *Hum Reprod* 16, 1866-1874.

Cles L D, Bruch K & Stamm W E. (1988) Staining characteristics of six commercially available monoclonal immunofluorescence reagents for direct diagnosis of *Chlamydia trachomatis* infections. *J Clin Microbiol* 26, 1735-1737.

Cohen C R, Manhart L E, Bukusi E A, Astete S, Brunham R C, Holmes K K, Sinei S K, Bwayo J J & Totten P A. (2002) Association between *Mycoplasma genitalium* and acute endometritis. *Lancet* 359, 765-766.

Cohen C R, Nosek M, Meier A, Astete S G, Iverson-Cabral S, Mugo N R & Totten P A. (2007) *Mycoplasma genitalium* infection and persistence in a cohort of female sex workers in Nairobi, Kenya. *Sex Transm Dis* 34, 274-279.

Cohn B A, Overstreet J W, Fogel R J, Brazil C K, Baird D D & Cirillo P M. (2002) Epidemiologic studies of human semen quality: considerations for study design. *Am J Epidemiol* 155, 664-671.

Cole B C, Piepkorn M W & Wright E C. (1985) Influence of genes of the major histocompatibility complex on ulcerative dermal necrosis induced in mice by Mycoplasma arthritidis. *J Invest Dermatol* 85, 357-361.

Comhaire F H. (1994) Male contraception: hormonal, mechanical and other. *Hum Reprod* 9, 586-590.

Comhaire F H, Mahmoud A M, Depuydt C E, Zalata A A & Christophe A B. (1999) Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's viewpoint. *Hum Reprod Update* 5, 393-398.

Coppus S F, Opmeer B C, Logan S, van der Veen F, Bhattacharya S & Mol B W. (2007) The predictive value of medical history taking and Chlamydia IgG ELISA antibody testing (CAT) in the selection of subfertile women for diagnostic laparoscopy: a clinical prediction model approach. *Hum Reprod* 22, 1353-1358.

Czuppon A B. (1984) Biochemical characterization of a human spermatozoal sialoglycoprotein with respect to antigenicity masking by its sialic acid moieties. *Biochem Int* 8, 9-18.

Dabekausen Y A, Evers J L, Land J A & Stals F S. (1994) *Chlamydia trachomatis* antibody testing is more accurate than hysterosalpingography in predicting tubal factor infertility. *Fertil Steril* 61, 833-837.

de Barbeyrac B, Papaxanthos-Roche A, Mathieu C, Germain C, Brun J L, Gachet M, Mayer G, Bebear C, Chene G & Hocke C. (2006) *Chlamydia trachomatis* in subfertile couples undergoing an in vitro fertilization program: a prospective study. *Eur J Obstet Gynecol Reprod Biol* 129, 46-53.

de Lima Freitas N S, Borborema-Santos C M, Barroso Serrao das Neves D, Costa de Oliveira C M, Dutra Ferreira J R & Astolfi-Filho S. (2011) High prevalence detection of *Chlamydia trachomatis* by polymerase chain reaction in endocervical samples of infertile women attending university hospital in Manaus-Amazonas, Brazil. *Gynecol Obstet Invest* 72, 220-226.

de Vries R, van Bergen J E, de Jong-van den Berg L T & Postma M J. (2006) Systematic screening for Chlamydia trachomatis: estimating cost-effectiveness using dynamic modeling and Dutch data. *Value in health : the journal of the International Society for Pharmacoeconomics and Outcomes Research* 9, 1-11.

den Hartog J E, Morre S A & Land J A. (2006) *Chlamydia trachomatis*-associated tubal factor subfertility: Immunogenetic aspects and serological screening. *Hum Reprod Update* 12, 719-730.

Depuydt C, Zalata A, Christophe A, Mahmoud A & Comhaire F. (1998) Mechanisms of sperm deficiency in male accessory gland infection. *Andrologia* 30 Suppl 1, 29-33.

Dieterle S, Mahony J B, Luinstra K E & Stibbe W. (1995) Chlamydial immunoglobulin IgG and IgA antibodies in serum and semen are not associated with the presence of Chlamydia trachomatis DNA or rRNA in semen from male partners of infertile couples. *Hum Reprod* 10, 315-319.

Domes T, Lo K C, Grober E D, Mullen J B, Mazzulli T & Jarvi K. (2012) The utility and cost of *Chlamydia trachomatis* and Neisseria gonorrhoeae screening of a male infertility population. *Fertil Steril* 97, 299-305.

Dorey M D, Choi Y H, Soldan K & Vynnycky E. (2012) Modelling the effect of Chlamydia trachomatis testing on the prevalence of infection in England: what impact can we expect from the National Chlamydia Screening Programme? *Sex Transm Infect*.

Edwards R K, Ferguson R J, Reyes L, Brown M, Theriaque D W & Duff P. (2006) Assessing the relationship between preterm delivery and various microorganisms recovered from the lower genital tract. *J Matern Fetal Neona* 19, 357-363.

Eggert-Kruse W, Boit R, Rohr G, Aufenanger J, Hund M & Strowitzki T. (2001) Relationship of seminal plasma interleukin (IL) -8 and IL-6 with semen quality. *Hum Reprod* 16, 517-528.

Eggert-Kruse W, Gerhard I, Naher H, Tilgen W & Runnebaum B. (1990) Chlamydial infection--a female and/or male infertility factor? *Fertil Steril* 53, 1037-1043.

Eggert-Kruse W, Neuer A, Clussmann C, Boit R, Geissler W, Rohr G & Strowitzki T. (2002) Seminal antibodies to human 60kd heat shock protein (HSP 60) in male partners of subfertile couples. *Hum Reprod* 17, 726-735.

Eggert-Kruse W, Rohr G, Bockem-Hellwig S, Huber K, Christmann-Edoga M & Runnebaum B. (1995) Immunological aspects of subfertility. *Int J Androl* 18 Suppl 2, 43-52.

Eggert-Kruse W, Rohr G, Demirakca T, Rusu R, Naher H, Petzoldt D & Runnebaum B. (1997) Chlamydial serology in 1303 asymptomatic subfertile couples. *Hum Reprod* 12, 1464-1475.

Eggert-Kruse W, Rohr G, Kunt B, Meyer A, Wondra J, Strowitzki T & Petzoldt D. (2003) Prevalence of *Chlamydia trachomatis* in subfertile couples. *Fertil Steril* 80, 660-663.

Eggert-Kruse W, Weltin M & Strowitzki T. (2011) Are chlamydial lipopolysaccharide-directed antibodies in seminal plasma or serum clinically significant during investigation of male infertility? *Urology* 77, 1101-1106.

Ehret J M & Judson F N. (1989) Genital Chlamydia infections. *Clin Lab Med* 9, 481-500.

Eisenberg M L, Lathi R B, Baker V L, Westphal L M, Milki A A & Nangia A K. (2012) The frequency of the male infertility evaluation: data from the National Survey of Family Growth. *J Urol*.

El Hakim E A, Gordon U D & Akande V A. (2010) The relationship between serum Chlamydia antibody levels and severity of disease in infertile women with tubal damage. *Arch Gynecol Obstet* 281, 727-733.

Eley A, Hosseinzadeh S, Hakimi H, Geary I & Pacey A A. (2005) Apoptosis of ejaculated human sperm is induced by co-incubation with *Chlamydia trachomatis* lipopolysaccharide. *Hum Reprod* 20, 2601-2607.

Eley A, Oxley K M, Spencer R C, Kinghorn G R, Ben-Ahmeida E T & Potter C W. (1992) Detection of *Chlamydia trachomatis* by the polymerase chain reaction in young patients with acute epididymitis. *Eur J Clin Microbiol Infect Dis* 11, 620-623.

Eley A & Pacey A A. (2011) The value of testing semen for *Chlamydia trachomatis* in men of infertile couples. *Int J Androl* 34, 391-401.

Eley A, Pacey A A, Galdiero M & Galdiero F. (2005) Can *Chlamydia trachomatis* directly damage your sperm? *Lancet Infect Dis* 5, 53-57.

Equils O, Lu D, Gatter M, Witkin S S, Bertolotto C, Arditi M, McGregor J A, Simmons C F & Hobel C J. (2006) Chlamydia heat shock protein 60 induces trophoblast apoptosis through TLR4. *J Immunol* 177, 1257-1263.

Erbengi T. (1993) Ultrastructural observations on the entry of *Chlamydia trachomatis* into human spermatozoa. *Hum Reprod* 8, 416-421.

Eschenbach D A. (1984) Acute pelvic inflammatory disease. *Urol Clin North Am* 11, 65-81.

Evenson D P, Darzynkiewicz Z & Melamed M R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 210, 1131-1133.

Everett K D & Andersen A A. (1999) Identification of nine species of the Chlamydiaceae using PCR-RFLP. *Int J Syst Bacteriol* 49 Pt 2, 803-813.

Everett K D, Bush R M & Andersen A A. (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* 49 Pt 2, 415-440.

Falk L, Fredlund H & Jensen J S. (2005) Signs and symptoms of urethritis and cervicitis among women with or without *Mycoplasma genitalium* or *Chlamydia trachomatis* infection. *Sex Transm Infect* 81, 73-78.

Fanchin R, Harmas A, Benaoudia F, Lundkvist U, Olivennes F & Frydman R. (1998) Microbial flora of the cervix assessed at the time of embryo transfer adversely affects in vitro fertilization outcome. *Fertil Steril* 70, 866-870.

Farhi J & Ben-Haroush A. (2011) Distribution of causes of infertility in patients attending primary fertility clinics in Israel. *Isr Med Assoc J* 13, 51-54

Fatholahzadeh B, Bahador A, Haghighi - Hassanabad M, Bazarjani F, Haghighi F. (2012) Comparative Screening of Chlamydia Trachomatis Infection in Women Population in Tehran, Iran. *Iran Red Crescent Med J* 14, 289-293.

Fauser B C (2004) Revised 2003 consensus on diagnostic criteria and longterm health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod* 19, 41-47.

Fawaz F S, van Ooij C, Homola E, Mutka S C & Engel J N. (1997) Infection with *Chlamydia trachomatis* alters the tyrosine phosphorylation and/or localization of several host cell proteins including cortactin. *Infect Immun* 65, 5301-5308.Fenton K A & Ward H. (2004) National chlamydia screening programme in England: making progress. *Sex Transm Infect* 80, 331-333.

Fernandez-Gonzalez R, Moreira P N, Perez-Crespo M, Sanchez-Martin M, Ramirez M A, Pericuesta E, Bilbao A, Bermejo-Alvarez P, Hourcade J D, de Fonseca F R & Gutierrez-Adan A. (2008) Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol Reprod* 78, 761-772.

Fernandez-Real J M, Lopez-Bermejo A, Vendrell J, Ferri M J, Recasens M & Ricart W. (2006) Burden of infection and insulin resistance in healthy middle-aged men. *Diabetes Care* 29, 1058-1064.

Fraser C M, Gocayne J D, White O, Adams M D, Clayton R A, Fleischmann R D, Bult C J, Kerlavage A R, Sutton G, Kelley J M, Fritchman R D, Weidman J F, Small K V, Sandusky M, Fuhrmann J, Nguyen D, Utterback T R, Saudek D M,

Phillips C A, Merrick J M, Tomb J F, Dougherty B A, Bott K F, Hu P C, Lucier T S, Peterson S N, Smith H O, Hutchison C A, 3rd & Venter J C. (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* 270, 397-403.

Fresse A S, Sueur J M & Hamdad F. (2010) Diagnosis and follow-up of genital chlamydial infection by direct methods and by detection of serum IgG, IgA and secretory IgA. *Indian J Med Microbiol* 28, 326-331.

Friberg J & Fullan N. (1983) Attachment of Escherichia coli to human spermatozoa. *Am J Obstet Gynecol* 146, 465-467.

Furuya Y, Akashi T & Fuse H. (2003) Soluble Fas and interleukin-6 and interleukin-8 levels in seminal plasma of infertile men. *Arch Androl* 49, 449-452.

Gallegos-Avila G, Alvarez-Cuevas S, Niderhauser-Garcia A, Ancer-Rodriguez J, Jaramillo-Rangel G & Ortega-Martinez M. (2009) Phagocytosis of spermatozoa and leucocytes by epithelial cells of the genital tract in infertile men infected with *Chlamydia trachomatis* and mycoplasmas. *Histopathology* 55, 232-234.

Gallegos G, Ramos B, Santiso R, Goyanes V, Gosalvez J & Fernandez J L. (2008) Sperm DNA fragmentation in infertile men with genitourinary infection by *Chlamydia trachomatis* and Mycoplasma. *Fertil Steril* 90, 328-334.

Garnett G P. (2008) How much infertility does chlamydia cause? *Sex Transm Infect* 84, 157-158.

Gdoura R, Kchaou W, Ammar-Keskes L, Chakroun N, Sellemi A, Znazen A, Rebai T & Hammami A. (2008) Assessment of *Chlamydia trachomatis, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis*, and *Mycoplasma genitalium* in semen and first void urine specimens of asymptomatic male partners of infertile couples. *J Androl* 29, 198-206.

Gdoura R, Kchaou W, Chaari C, Znazen A, Keskes L, Rebai T & Hammami A. (2007) *Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infect Dis* 7, 129.

Geisler W M, Black C M, Bandea C I & Morrison S G. (2008) *Chlamydia trachomatis* OmpA genotyping as a tool for studying the natural history of genital chlamydial infection. *Sex Transm Infect* 84, 541-544; discussion 544-545.

Gervassi A L, Grabstein K H, Probst P, Hess B, Alderson M R & Fling S P. (2004) Human CD8+ T cells recognize the 60-kDa cysteine-rich outer membrane protein from *Chlamydia trachomatis*. *J Immunol* 173, 6905-6913.

Ghanaat J, Afshari J T, Ghazvini K & Malvandi M. (2008) Prevalence of genital Chlamydia in Iranian males with urethritis attending clinics in Mashhad. *East Mediterr Health J* 14, 1333-1337.

Ghazal-Aswad S, Badrinath P, Osman N, Abdul-Khaliq S, Mc Ilvenny S & Sidky I. (2004) Prevalence of *Chlamydia trachomatis* infection among women in a Middle Eastern community. *BMC Womens Health* 4, 3.

Ghazvini K, Ahmadnia H & Ghanaat J. (2012) Frequency of Chlamydia trachomatis among male patients with urethritis in northeast of Iran detected by polymerase chain reaction. *Saudi J Kidney Dis Transpl* 23, 316-320.

Gillespie P, O'Neill C, Adams E, Turner K, O'Donovan D, Brugha R, Vaughan D, O'Connell E, Cormican M, Balfe M, Coleman C, Fitzgerald M & Fleming C. (2012) The cost and cost-effectiveness of opportunistic screening for Chlamydia trachomatis in Ireland. *Sex Transm Infect* 88, 222-228.

Glass J I, Assad-Garcia N, Alperovich N, Yooseph S, Lewis M R, Maruf M, Hutchison C A, 3rd, Smith H O & Venter J C. (2006) Essential genes of a minimal bacterium. *Proc Natl Acad Sci U S A* 103, 425-430.

Gonzales G F, Munoz G, Sanchez R, Henkel R, Gallegos-Avila G, Diaz-Gutierrez O, Vigil P, Vasquez F, Kortebani G, Mazzolli A & Bustos-Obregon E. (2004) Update on the impact of *Chlamydia trachomatis* infection on male fertility. *Andrologia* 36, 1-23.

Gordon F B & Quan A L. (1965) Isolation of the Trachoma Agent in Cell Culture. *Proc Soc Exp Biol Med* 118, 354-359.

Gorga F, Galdiero M, Buommino E & Galdiero E. (2001) Porins and lipopolysaccharide induce apoptosis in human spermatozoa. *Clin Diagn Lab Immunol* 8, 206-208.

Gravett M G, Hummel D, Eschenbach D A & Holmes K K. (1986) Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstet Gynecol* 67, 229-237.

Gravett M G, Nelson H P, DeRouen T, Critchlow C, Eschenbach D A & Holmes K K. (1986) Independent associations of bacterial vaginosis and *Chlamydia trachomatis* infection with adverse pregnancy outcome. *Jama* 256, 1899-1903.

Grayston J T, Kuo C C, Wang S P & Altman J. (1986) A new Chlamydia psittaci strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 315, 161-168.

Grzesko J, Elias M, Maczynska B, Kasprzykowska U, Tlaczala M & Goluda M. (2009) Occurrence of *Mycoplasma genitalium* in fertile and infertile women. *Fertil Steril* 91, 2376-2380.

Guerra-Infante F M, Tapia-Yanez J R, Lopez-Hurtado M, Flores-Medina S & Diaz-Garcia F J. (2005) [Chlamydia trachomatis infection in men and its association with gynecologic alterations in their sexual partners]. *Rev Invest Clin* 57, 406-414.

Haggerty C L, Gottlieb S L, Taylor B D, Low N, Xu F & Ness R B. (2010) Risk of sequelae after *Chlamydia trachomatis* genital infection in women. *J Infect Dis* 201 Suppl 2, S134-155.

Haggerty C L, Totten P A, Astete S G & Ness R B. (2006) *Mycoplasma genitalium* among women with nongonococcal, nonchlamydial pelvic inflammatory disease. *Infect Dis Obstet Gynecol* 2006, 30184.

Haghighi Hasanabad M, Mohammadzadeh M, Bahador A, Fazel N, Rakhshani H & Majnooni A. (2011) Prevalence of *Chlamydia trachomatis* and Mycoplasma genitalium in pregnant women of Sabzevar-Iran. *Iran J Microbiol* 3, 123-128.

Hakimi H, Geary I, Pacey A & Eley A. (2006) Spermicidal activity of bacterial lipopolysaccharide is only partly due to lipid A. *J Androl* 27, 774-779.

Hamdad-Daoudi F, Petit J & Eb F. (2004) Assessment of *Chlamydia trachomatis* infection in asymptomatic male partners of infertile couples. *J Med Microbiol* 53, 985-990.

Hatch T P. (1996) Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? *J Bacteriol* 178, 1-5.

Henderson B & Wilson M. (1996) Cytokine induction by bacteria: beyond lipopolysaccharide. *Cytokine* 8, 269-282.

Hitti J, Garcia P, Totten P, Paul K, Astete S & Holmes K K. (2010) Correlates of cervical *Mycoplasma genitalium* and risk of preterm birth among Peruvian women. *Sex Transm Dis* 37, 81-85.

Hogdahl M & Kihlstrom E. (2007) Leucocyte esterase testing of first-voided urine and urethral and cervical smears to identify *Mycoplasma genitalium*-infected men and women. *Int J STD AIDS* 18, 835-838.

Hollegaard S, Vogel I, Thorsen P, Jensen I P, Mordhorst C H & Jeune B. (2007) *Chlamydia trachomatis* C-complex serovars are a risk factor for preterm birth. *In Vivo* 21, 107-112.

Holmes K K, Handsfield H H, Wang S P, Wentworth B B, Turck M, Anderson J B & Alexander E R. (1975) Etiology of nongonococcal urethritis. *N Engl J Med* 292, 1199-1205.

Horn M, Collingro A, Schmitz-Esser S, Beier C L, Purkhold U, Fartmann B, Brandt P, Nyakatura G J, Droege M, Frishman D, Rattei T, Mewes H W & Wagner M. (2004) Illuminating the evolutionary history of chlamydiae. *Science* 304, 728-730.

Hosseinzadeh S, Brewis I A, Eley A & Pacey A A. (2001) Co-incubation of human spermatozoa with *Chlamydia trachomatis* serovar E causes premature sperm death. *Hum Reprod* 16, 293-299.

Hosseinzadeh S, Brewis I A, Pacey A A, Moore H D & Eley A. (2000) Coincubation of human spermatozoa with *Chlamydia trachomatis* in vitro causes increased tyrosine phosphorylation of sperm proteins. *Infect Immun* 68, 4872-4876.

Hosseinzadeh S, Eley A & Pacey A A. (2004) Semen quality of men with asymptomatic chlamydial infection. *J Androl* 25, 104-109.

Hosseinzadeh S, Pacey A A & Eley A. (2003) *Chlamydia trachomatis*-induced death of human spermatozoa is caused primarily by lipopolysaccharide. *J Med Microbiol* 52, 193-200.

Hu D, Hook E W, 3rd & Goldie S J. (2004) Screening for Chlamydia trachomatis in women 15 to 29 years of age: a cost-effectiveness analysis. *Ann Intern Med* 141, 501-513.

Hu D, Hook E W, 3rd & Goldie S J. (2006) The impact of natural history parameters on the cost-effectiveness of Chlamydia trachomatis screening strategies. *Sex Transm Dis* 33, 428-436.

Huber H, Hohn M J, Rachel R, Fuchs T, Wimmer V C & Stetter K O. (2002) A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* 417, 63-67.

Idahl A, Abramsson L, Kumlin U, Liljeqvist J A & Olofsson J I. (2007) Male serum *Chlamydia trachomatis* IgA and IgG, but not heat shock protein 60 IgG, correlates with negatively affected semen characteristics and lower pregnancy rates in the infertile couple. *Int J Androl* 30, 99-107.

Idahl A, Boman J, Kumlin U & Olofsson J I. (2004) Demonstration of *Chlamydia trachomatis* IgG antibodies in the male partner of the infertile couple is correlated with a reduced likelihood of achieving pregnancy. *Hum Reprod* 19, 1121-1126.

Ikeme A C, Ezegwui H U, Ikeako L C, Agbata I & Agbata E. (2011) Seroprevalence of Chlamydia trachomatis in Enugu, Nigeria. *Niger J Clin Pract* 14, 176-180.

Imai H, Nakao H, Shinohara H, Fujii Y, Tsukino H, Hamasuna R, Osada Y, Fukushima K, Inamori M, Ikenoue T & Katoh T. (2010) Population-based study of asymptomatic infection with *Chlamydia trachomatis* among female and male students. *Int J STD AIDS* 21, 362-366.

Ivanov I B, Kuzmin M D & Gritsenko V A. (2009) Microflora of the seminal fluid of healthy men and men suffering from chronic prostatitis syndrome. *Int J Androl* 32, 462-467.

Iverson-Cabral S L, Manhart L E & Totten P A. (2011) Detection of *Mycoplasma genitalium*-reactive cervicovaginal antibodies among infected women. *Clin Vaccine Immunol* 18, 1783-1786.

Jalal H, Al-Suwaine A, Stephen H, Carne C & Sonnex C. (2007) Comparative performance of the Roche COBAS Amplicor assay and an in-house real-time PCR assay for diagnosis of *Chlamydia trachomatis* infection. *J Med Microbiol* 56, 320-322.

Jalal H, Stephen H, Alexander S, Carne C & Sonnex C. (2007) Development of real-time PCR assays for genotyping of *Chlamydia trachomatis*. *J Clin Microbiol* 45, 2649-2653.

Jensen J S. (2006) *Mycoplasma genitalium* infections. Diagnosis, clinical aspects, and pathogenesis. *Dan Med Bull* 53, 1-27.

Jensen J S, Blom J & Lind K. (1994) Intracellular location of *Mycoplasma genitalium* in cultured Vero cells as demonstrated by electron microscopy. *Int J Exp Pathol* 75, 91-98.

Jensen J S, Borre M B & Dohn B. (2003) Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. *J Clin Microbiol* 41, 261-266.

Jensen J S, Orsum R, Dohn B, Uldum S, Worm A M & Lind K. (1993) *Mycoplasma genitalium*: a cause of male urethritis? *Genitourin Med* 69, 265-269.

Jensen J S, Uldum S A, Sondergard-Andersen J, Vuust J & Lind K. (1991) Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J Clin Microbiol* 29, 46-50.

Johnson A M & Horner P. (2008) A new role for *Chlamydia trachomatis* serology? *Sex Transm Infect* 84, 79-80.

Joki-Korpela P, Sahrakorpi N, Halttunen M, Surcel H M, Paavonen J & Tiitinen A. (2009) The role of *Chlamydia trachomatis* infection in male infertility. *Fertil Steril* 91, 1448-1450.

Joseph A. (2011) Role of Minimal Access Techniques in Tubal Factor Inferitility. *World journal of Laparoscopic Surgery* 4, 59-66.

Joyner J L, Douglas J M, Jr., Foster M & Judson F N. (2002) Persistence of *Chlamydia trachomatis* infection detected by polymerase chain reaction in untreated patients. *Sex Transm Dis* 29, 196-200.

Jurstrand M, Jensen J S, Magnuson A, Kamwendo F & Fredlund H. (2007) A serological study of the role of *Mycoplasma genitalium* in pelvic inflammatory disease and ectopic pregnancy. *Sex Transm Infect* 83, 319-323.

Karinen L, Pouta A, Bloigu A, Koskela P, Paldanius M, Leinonen M, Saikku P, Jervelin M R & Hartikainen A L. (2005) Serum C-reactive protein and Chlamydia trachomatis antibodies in preterm delivery. *Obstet Gynecol* 106, 73-80.

Katz B P, Thom S, Blythe M J, Arno J N, Caine V M & Jones R B. (1994) Fertility in Adolescent Women Previously Treated for Genitourinary Chlamydial Infection. *Adolescent Pediat G* 7, 147-152.

Keane F E A, Thomas B J, Gilroy C B, Renton A & Taylor-Robinson D. (2000) The association of Mycoplasma hominis, Ureaplasma urealyticum and Mycoplasma genitalium with bacterial vaginosis: observations on heterosexual women and their male partners. *Int J STD AIDS* 11, 356-360.

Keck C, Gerber-Schafer C, Clad A, Wilhelm C & Breckwoldt M. (1998) Seminal tract infections: impact on male fertility and treatment options. *Hum Reprod Update* 4, 891-903.

Kelly K A & Rank R G. (1997) Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with *Chlamydia trachomatis*. *Infect Immun* 65, 5198-5208.

Kennedy S, Bergqvist A, Chapron C, D'Hooghe T, Dunselman G, Greb R, Hummelshoj L, Prentice A & Saridogan E. (2005) ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum Reprod* 20, 2698-2704.

Khalaf Y. (2003) ABC of subfertility. Tubal subfertility. *Bmj* 327, 610-613.

Kidd S A, Eskenazi B & Wyrobek A J. (2001) Effects of male age on semen quality and fertility: a review of the literature. *Fertil Steril* 75, 237-248.

Kjaergaard N, Kristensen B, Hansen E S, Farholt S, Schonheyder H C, Uldbjerg N & Madsen H. (1997) Microbiology of semen specimens from males attending a fertility clinic. *Apmis* 105, 566-570.

Kokab A, Akhondi M M, Sadeghi M R, Modarresi M H, Aarabi M, Jennings R, Pacey A A & Eley A. (2010) Raised inflammatory markers in semen from men with asymptomatic chlamydial infection. *J Androl* 31, 114-120.

Kopa Z, Wenzel J, Papp G K & Haidl G. (2005) Role of granulocyte elastase and interleukin-6 in the diagnosis of male genital tract inflammation. *Andrologia* 37, 188-194.

Kruger T F & Coetzee K. (1999) The role of sperm morphology in assisted reproduction. *Hum Reprod Update* 5, 172-178.

Kuo C C, Wentwort.Bb, Wang S P & Grayston J T. (1972) Primary Isolation of Tric Organisms in Hela 229 Cells Treated with Deae-Dextran. *Journal of Infectious Diseases* 125, 665-&.

La Vignera S, Vicari E, Condorelli R A, D'Agata R & Calogero A E. (2011) Male accessory gland infection and sperm parameters (review). *Int J Androl* 34, e330-347.

Labbe A C, Frost E, Deslandes S, Mendonca A P, Alves A C & Pepin J. (2002) *Mycoplasma genitalium* is not associated with adverse outcomes of pregnancy in Guinea-Bissau. *Sex Transm Infect* 78, 289-291.

Lan J, Ossewaarde J M, Walboomers J M, Meijer C J & van den Brule A J. (1994) Improved PCR sensitivity for direct genotyping of *Chlamydia trachomatis* serovars by using a nested PCR. *J Clin Microbiol* 32, 528-530.

Land J A, Van Bergen J E, Morre S A & Postma M J. (2010) Epidemiology of Chlamydia trachomatis infection in women and the cost-effectiveness of screening. *Hum Reprod Update* 16, 189-204.

Larson K L, DeJonge C J, Barnes A M, Jost L K & Evenson D P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod* 15, 1717-1722.

Lee H H, Chernesky M A, Schachter J, Burczak J D, Andrews W W, Muldoon S, Leckie G & Stamm W E. (1995) Diagnosis of *Chlamydia trachomatis* genitourinary infection in women by ligase chain reaction assay of urine. *Lancet* 345, 213-216.

Leung P H M, Boost M V, Lau J T F, Wong A T Y, Pang M, Ng T K & Tong E T F. (2009) Prevalence and risk factors for *Chlamydia trachomatis* infection among cross-border truck drivers in Hong Kong. *Sex Transm Infect* 85, 27-29.

Lind K. (1982) Serological cross-reactions between "*Mycoplasma genitalium*" and M. pneumoniae. *Lancet* 2, 1158-1159.

Low N. (2007) Screening programmes for chlamydial infection: when will we ever learn? *Bmj* 334, 725-728.

Low N, Broutet N, Adu-Sarkodie Y, Barton P, Hossain M & Hawkes S. (2006) Global control of sexually transmitted infections. *Lancet* 368, 2001-2016.

Low N, Egger M, Sterne J A C, Harbord R M, Ibrahim F, Lindblom B & Herrmann B. (2006) Incidence of severe reproductive tract complications associated with

diagnosed genital chlamydial infection: the Uppsala Women's Cohort Study. *Sex Transm Infect* 82, 212-218.

Luki N, Lebel P, Boucher M, Doray B, Turgeon J & Brousseau R. (1998) Comparison of polymerase chain reaction assay with culture for detection of genital mycoplasmas in perinatal infections. *Eur J Clin Microbiol Infect Dis* 17, 255-263.

Luo D, Xu W, Liang G, Wang S, Wang Z, Bi Z & Zhu W. (1999) Isolation and identification of *Mycoplasma genitalium* from high risk populations of sexually transmitted diseases in China. *Chin Med J (Engl)* 112, 489-492.

Magee D M, Smith J G, Bleicker C A, Carter C J, Bonewald L F, Schachter J & Williams D M. (1992) *Chlamydia trachomatis* pneumonia induces in vivo production of interleukin-1 and -6. *Infect Immun* 60, 1217-1220.

Mahmud N U, Hossain M A, Nahar K, Mahmud C, Paul S K, Ahmed S, Haque N, Kalam A & Parveen U S. (2011) Seroprevalence of genital *Chlamydia trachomatis* infection in women of reproductive age. *Mymensingh Med J* 20, 187-191.

Manavi K. (2006) A review on infection with *Chlamydia trachomatis*. *Best Pract Res Clin Obstet Gynaecol* 20, 941-951.

Manhart L E, Critchlow C W, Holmes K K, Dutro S M, Eschenbach D A, Stevens C E & Totten P A. (2003) Mucopurulent cervicitis and *Mycoplasma genitalium*. *J Infect Dis* 187, 650-657.

Marconi M, Pilatz A, Wagenlehner F, Diemer T & Weidner W. (2009) Impact of infection on the secretory capacity of the male accessory glands. *Int Braz J Urol* 35, 299-308; discussion 308-299.

Mardh P A. (2004) Tubal factor infertility, with special regard to chlamydial salpingitis. *Curr Opin Infect Dis* 17, 49-52.

Mardh P A, Colleen S & Sylwan J. (1980) Inhibitory effect on the formation of chlamydial inclusions in McCoy cells by seminal fluid and some of its components. *Invest Urol* 17, 510-513.

Mardh P A, Lind I, Svensson L, Westrom L & Moller B R. (1981) Antibodies to *Chlamydia trachomatis*, Mycoplasma hominis, and Neisseria gonorrhoeae in sera from patients with acute salpingitis. *Br J Vener Dis* 57, 125-129.

Marks E, Verolin M, Stensson A & Lycke N. (2007) Differential CD28 and inducible costimulatory molecule signaling requirements for protective CD4+ T-cell-mediated immunity against genital tract Chlamydia trachomatis infection. *Infect Immun* 75, 4638-4647.

Martinez-Prado E & Camejo Bermudez M I. (2010) Expression of IL-6, IL-8, TNF-alpha, IL-10, HSP-60, anti-HSP-60 antibodies, and anti-sperm antibodies, in semen of men with leukocytes and/or bacteria. *Am J Reprod Immunol* 63, 233-243.

Matalliotakis I, Arici A, Goumenou A, Koumantakis G, Selam B, Matalliotakis G & Koumantakis E. (2002) Distinct expression pattern of cytokines in semen of men with genital infection and oligo-terato-asthenozoospermia. *Am J Reprod Immunol* 48, 170-175.

Mavrov G I. (1995) [An electron microscopic study of the interaction of *Chlamydia trachomatis* with human spermatozoa]. *Mikrobiol Z* 57, 74-79.

McGowin C L & Anderson-Smits C. (2011) *Mycoplasma genitalium:* an emerging cause of sexually transmitted disease in women. *PLoS Pathog* 7, e1001324.

McGowin C L, Popov V L & Pyles R B. (2009) Intracellular *Mycoplasma genitalium* infection of human vaginal and cervical epithelial cells elicits distinct patterns of

inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing. *BMC Microbiol* 9, 139.

McGowin C L, Spagnuolo R A & Pyles R B. (2010) *Mycoplasma genitalium* rapidly disseminates to the upper reproductive tracts and knees of female mice following vaginal inoculation. *Infect Immun* 78, 726-736.

Michel C E, Sonnex C, Carne C A, White J A, Magbanua J P, Nadala E C, Jr. & Lee H H. (2007) *Chlamydia trachomatis* load at matched anatomic sites: implications for screening strategies. *J Clin Microbiol* 45, 1395-1402.

Miller W C, Ford C A, Morris M, Handcock M S, Schmitz J L, Hobbs M M, Cohen M S, Harris K M & Udry J R. (2004) Prevalence of chlamydial and gonococcal infections among young adults in the United States. *Jama* 291, 2229-2236.

Mirnejad R, Amirmozafari N & Kazemi B. (2011) Simultaneous and rapid differential diagnosis of *Mycoplasma genitalium* and Ureaplasma urealyticum based on a polymerase chain reaction-restriction fragment length polymorphism. *Indian J Med Microbiol* 29, 33-36.

Mitchell L A, De Iuliis G N & Aitken R J. (2011) The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* 34, 2-13.

Moi H, Reinton N & Moghaddam A. (2009) *Mycoplasma genitalium* in women with lower genital tract inflammation. *Sex Transm Infect* 85, 10-14.

Mol B W J, Dijkman B, Wertheim P, Lijmer J, vanderVeen F & Bossuyt P M M. (1997) The accuracy of serum chlamydial antibodies in the diagnosis of tubal pathology: A meta-analysis. *Fertil Steril* 67, 1031-1037.

Moller B R & Mardh P A. (1980) Experimental epididymitis and urethritis in grivet monkeys provoked by *Chlamydia trachomatis*. *Fertil Steril* 34, 275-279.

Moller B R, Taylor-Robinson D & Furr P M. (1984) Serological evidence implicating *Mycoplasma genitalium* in pelvic inflammatory disease. *Lancet* 1, 1102-1103.

Molyneux D H. (2004) "Neglected" diseases but unrecognised successes--challenges and opportunities for infectious disease control. *Lancet* 364, 380-383.

Moncada J, Chow J M & Schachter J. (2003) Volume effect on sensitivity of nucleic acid amplification tests for detection of *Chlamydia trachomatis* in urine specimens from females. *J Clin Microbiol* 41, 4842-4843.

Morin-Papunen L C, Duleba A J, Bloigu A, Jarvelin M R, Saikku P & Pouta A. (2010) Chlamydia antibodies and self-reported symptoms of oligo-amenorrhea and hirsutism: A new etiologic factor in polycystic ovary syndrome? *Fertil Steril* 94, 1799-1804.

Morre S A, van den Brule A J, Rozendaal L, Boeke A J, Voorhorst F J, de Blok S & Meijer C J. (2002) The natural course of asymptomatic *Chlamydia trachomatis* infections: 45% clearance and no development of clinical PID after one-year follow-up. *Int J STD AIDS* 13 Suppl 2, 12-18.

Morrison-Plummer J, Jones D H, Daly K, Tully J G, Taylor-Robinson D & Baseman J B. (1987) Molecular characterization of *Mycoplasma genitalium* species-specific and cross-reactive determinants: identification of an immunodominant protein of M. genitalium. *Isr J Med Sci* 23, 453-457.

Mpiga P, Mansour S, Morisset R, Beaulieu R & Ravaoarinoro M. (2006) Sustained interleukin-6 and interleukin-8 expression following infection with *Chlamydia trachomatis* serovar L2 in a HeLa/THP-1 cell co-culture model. *Scand J Immunol* 63, 199-207.

Mylonas I. (2012) Female genital Chlamydia trachomatis infection: where are we heading? *Arch Gynecol Obstet* 285, 1271-1285.
Namavar Jahromi B, Parsanezhad M E & Ghane-Shirazi R. (2001) Female genital tuberculosis and infertility. *Int J Gynaecol Obstet* 75, 269-272.
Nelson W G, De Marzo A M & Isaacs W B. (2003) Prostate cancer. *N Engl J Med* 349, 366-381.
Nijs M, Creemers E, Cox A, Franssen K, Janssen M, Vanheusden E, De Jonge C & Ombelet W. (2009) Chromomycin A3 staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. *Reprod Biomed Online* 19, 671-684.
Norman J. (2002) Epidemiology of female genital *Chlamydia trachomatis* infections. *Best Pract Res Clin Obstet Gynaecol* 16, 775-787.
Oakeshott P, Kerry S, Aghaizu A, Atherton H, Hay S, Taylor-Robinson D, Simms I & Hay P. (2010) Randomised controlled trial of screening for Chlamydia trachomatis to prevent pelvic inflammatory disease: the POPI (prevention of pelvic infection) trial. *Bmj* 340, c1642.
Ochsendorf F R, Ozdemir K, Rabenau H, Fenner T, Oremek R, Milbradt R & Doerr H W. (1999) Chlamydia trachomatis and male infertility: chlamydia-IgA antibodies in seminal plasma are *C. trachomatis* specific and associated with an inflammatory response. *J Eur Acad Dermatol Venereol* 12, 143-152.
Ouzounova-Raykova V, Ouzounova I & Mitov I G. (2010) May Chlamydia trachomatis be an aetiological agent of chronic prostatic infection? *Andrologia* 42, 176-181.
Paavonen J. (1995) [Chlamydia trachomatis infections: is screening cost-effective?]. *Laeknabladid* 81, 614-617.
Paavonen J & Eggert-Kruse W. (1999) *Chlamydia trachomatis*: impact on human reproduction. *Hum Reprod Update* 5, 433-447.
Paavonen J & Wolner-Hanssen P. (1989) *Chlamydia trachomatis*: a major threat to reproduction. *Hum Reprod* 4, 111-124.
Pace N R. (1997) A molecular view of microbial diversity and the biosphere. *Science* 276, 734-740.
Pacey A A. (2006) Is quality assurance in semen analysis still really necessary? A view from the andrology laboratory. *Hum Reprod* 21, 1105-1109.
Pacey A A & Eley A. (2004) *Chlamydia trachomatis* and male fertility. *Hum Fertil (Camb)* 7, 271-276.
Pacey A A. (2010) Environmental and lifestyle factors associated with sperm DNA damage. *Hum Fertil (Camb)* 13, 189-193. .
Palmer H M, Gilroy C B, Claydon E J & Taylor-Robinson D. (1991) Detection of *Mycoplasma genitalium* in the genitourinary tract of women by the polymerase chain reaction. *Int J STD AIDS* 2, 261-263.
Pannekoek Y, Trum J W, Bleker O P, van der Veen F, Spanjaard L & Dankert J. (2000) Cytokine concentrations in seminal plasma from subfertile men are not indicative of the presence of Ureaplasma urealyticum or Mycoplasma hominis in the lower genital tract. *J Med Microbiol* 49, 697-700.
Patel A, Rashid S, Godfrey E M & Panchal H. (2008) Prevalence of *Chlamydia trachomatis* and Neisseria gonorrhoeae genital infections in a publicly funded pregnancy termination clinic: empiric vs. indicated treatment? *Contraception* 78, 328-331.
Patel M A & Nyirjesy P. (2010) Role of Mycoplasma and ureaplasma species in female lower genital tract infections. *Curr Infect Dis Rep* 12, 417-422.

Patel R, Abbott M & Hicks D A. (1992) *Chlamydia trachomatis* detection. *Genitourin Med* 68, 65.

Penna G, Fibbi B, Amuchastegui S, Cossetti C, Aquilano F, Laverny G, Gacci M, Crescioli C, Maggi M & Adorini L. (2009) Human benign prostatic hyperplasia stromal cells as inducers and targets of chronic immuno-mediated inflammation. *J Immunol* 182, 4056-4064.

Pepin J, Labbe A C, Khonde N, Deslandes S, Alary M, Dzokoto A, Asamoah-Adu C, Meda H & Frost E. (2005) *Mycoplasma genitalium*: an organism commonly associated with cervicitis among west African sex workers. *Sex Transm Infect* 81, 67-72.

Peterson S N, Hu P C, Bott K F & Hutchison C A, 3rd. (1993) A survey of the *Mycoplasma genitalium* genome by using random sequencing. *J Bacteriol* 175, 7918-7930.

Peterson S N, Lucier T, Heitzman K, Smith E A, Bott K F, Hu P C & Hutchison C A, 3rd. (1995) Genetic map of the *Mycoplasma genitalium* chromosome. *J Bacteriol* 177, 3199-3204.

Piot P & Laga M. (1988) [Morbidity caused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in mothers and children in Kenya]. *Verh K Acad Geneeskd Belg* 50, 367-392.

Plastira K, Angelopoulou R, Mantas D, Msaouel P, Lyrakou S, Plastiras A, Bolaris S, Baka S & Paparisteidis N. (2007) The effects of age on the incidence of aneuploidy rates in spermatozoa of oligoasthenozoospermic patients and its relationship with ICSI outcome. *Int J Androl* 30, 65-72.

Practice Committee of American society for Reproductive Medicine (ASRM) (2006) The clinical utility of sperm DNA integrity testing. *Fertil Steril* 86, S35-S37

Pudney V A, Leese A M, Rickinson A B & Hislop A D. (2005) CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. *J Exp Med* 201, 349-360.

Punnonen R, Terho P, Nikkanen V & Meurman O. (1979) Chlamydial serology in infertile women by immunofluorescence. *Fertil Steril* 31, 656-659.

Quinn T C, Gaydos C, Shepherd M, Bobo L, Hook E W, 3rd, Viscidi R & Rompalo A. (1996) Epidemiologic and microbiologic correlates of *Chlamydia trachomatis* infection in sexual partnerships. *Jama* 276, 1737-1742.

Rajasekaran M, Hellstrom W J, Naz R K & Sikka S C. (1995) Oxidative stress and interleukins in seminal plasma during leukocytospermia. *Fertil Steril* 64, 166-171.

Ramsey K H & Rank R G. (1991) Resolution of chlamydial genital infection with antigen-specific T-lymphocyte lines. *Infect Immun* 59, 925-931.

Razin S, Yogev D & Naot Y. (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 62, 1094-1156.

Ripa K T. (1982) Microbiological Diagnosis of *Chlamydia Trachomatis* Infection. *Infection* 10, S19-S24.

Ross M J, Fan C, Ross M D, Chu T H, Shi Y, Kaufman L, Zhang W, Klotman M E & Klotman P E. (2006) HIV-1 infection initiates an inflammatory cascade in human renal tubular epithelial cells. *J Acquir Immune Defic Syndr* 42, 1-11.

Saiki R K, Scharf S, Faloona F, Mullis K B, Horn G T, Erlich H A & Arnheim N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.

Salari M H & Karimi A. (2003) Prevalence of Ureaplasma urealyticum and *Mycoplasma genitalium* in men with non-gonococcal urethritis. *East Mediterr Health J* 9, 291-295.

Saleh R A, Agarwal A, Nelson D R, Nada E A, El-Tonsy M H, Alvarez J G, Thomas A J, Jr. & Sharma R K. (2002) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil Steril* 78, 313-318.

Samra Z, Borin M, Bukowsky Y, Lipshitz Y & Sompolinsky D. (1988) Non-occurrence of *Mycoplasma genitalium* in clinical specimens. *Eur J Clin Microbiol Infect Dis* 7, 49-51.

Samra Z & Gadba R. (1993) Diagnosis of *Mycoplasma pneumoniae* infection by specific IgM antibodies using a new capture-enzyme-immunoassay. *Eur J Epidemiol* 9, 97-99.

Sasaki Y, Shintani M, Shimada T, Watanabe H & Sasaki T. (1992) Detection and discrimination of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* by the in vitro DNA amplification. *Microbiol Immunol* 36, 21-27.

Satta A, Stivala A, Garozzo A, Morello A, Perdichizzi A, Vicari E, Salmeri M & Calogero A E. (2006) Experimental *Chlamydia trachomatis* infection causes apoptosis in human sperm. *Hum Reprod* 21, 134-137.

Schachter J. (1999) Which test is best for chlamydia? *Curr Opin Infect Dis* 12, 41-45.

Schachter J. (2001) NAATs to diagnose *Chlamydia trachomatis* genital infection: a promise still unfulfilled. *Expert Rev Mol Diagn* 1, 137-144.

Schachter J, Banks J, Sugg N, Sung M, Storz J & Meyer K F. (1974) Serotyping of Chlamydia. I. Isolates of ovine origin. *Infect Immun* 9, 92-94.

Schachter J, Chow J M, Howard H, Bolan G & Moncada J. (2006) Detection of *Chlamydia trachomatis* by nucleic acid amplification testing: our evaluation suggests that CDC-recommended approaches for confirmatory testing are ill-advised. *J Clin Microbiol* 44, 2512-2517.

Schachter J & Wyrick P B. (1994) Culture and isolation of *Chlamydia trachomatis*. *Methods Enzymol* 236, 377-390.

Schoenwald E, Schmidt B L, Steinmetz G, Hosmann J, Pohla-Gubo G, Luger A & Gasser G. (1988) Diagnosis of *Chlamydia trachomatis* infection--culture versus serology. *Eur J Epidemiol* 4, 75-82.

Scholes D, Holmes K K, Stergachis A & Stamm W E. (1996) Screening for chlamydia to prevent pelvic inflammatory disease - Reply. *New Engl J Med* 335, 1532-1533.

Schulte R T, Ohl D A, Sigman M & Smith G D. (2010) Sperm DNA damage in male infertility: etiologies, assays, and outcomes. *J Assist Reprod Genet* 27, 3-12.

Sharma R K, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A & Agarwal A. (2010) TUNEL as a test for sperm DNA damage in the evaluation of male infertility. *Urology* 76, 1380-1386.

Shaw R W, Stanton S l. (2003) Gyanaecology.Third edition.

Shen H M, Dai J, Chia S E, Lim A & Ong C N. (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 17, 1266-1273.

Shimoya K, Matsuzaki N, Ida N, Okada T, Taniguchi T, Sawai K, Itoh S, Ohashi K, Sajl F & Tanizawa O. (1995) Detection of Monocyte Chemotactic and Activating Factor (Mcaf) and Interleukin (Il)-6 in Human Seminal Plasma and Effect of Leukospermia on These Cytokine Levels. *Am J Reprod Immunol* 34, 311-316.

Sigman M & Lopes L. (1993) The correlation between round cells and white blood cells in the semen. *J Urol* 149, 1338-1340.

SIGN. (2009) Management of genital *Chlamydia trachomatis* infection. *Scotish Intercollegiate Guidelines Network*.

Simms I, Eastick K, Mallinson H, Thomas K, Gokhale R, Hay P, Herring A & Rogers P A. (2003) Associations between *Mycoplasma genitalium*, *Chlamydia trachomatis* and pelvic inflammatory disease. *J Clin Pathol* 56, 616-618.

Simms I, Vickers M R, Stephenson J, Rogers P A & Nicoll A. (2000) National assessment of PID diagnosis, treatment and management in general practice: England and Wales. *Int J STD AIDS* 11, 440-444.

Skidmore S, Horner P & Mallinson H. (2006) Testing specimens for *Chlamydia trachomatis*. *Sex Transm Infect* 82, 272-275.

Soffer Y, Ronel R, Golan A, Herman A, Caspi E & Samra Z. (1990) Male Genital Mycoplasmas and *Chlamydia-Trachomatis* Culture - Its Relationship with Accessory-Gland Function, Sperm Quality, and Autoimmunity. *Fertil Steril* 53, 331-336.

Stamm W E. (1999) Chlamydia trachomatis infections: progress and problems. *J Infect Dis* 179 Suppl 2, S380-383.

Stamm W E, Tam M, Koester M & Cles L. (1983) Detection of *Chlamydia trachomatis* inclusions in Mccoy cell cultures with fluorescein-conjugated monoclonal antibodies. *J Clin Microbiol* 17, 666-668.

Stamm W E, Wagner K F, Amsel R, Alexander E R, Turck M, Counts G W & Holmes K K. (1980) Causes of the acute urethral syndrome in women. *N Engl J Med* 303, 409-415.

Stasiewicz D, Staroslawska E, Brzozowska A, Mocarska A, Losicki M, Szumilo J & Burdan F. (2012) [Epidemiology and risk factors of the prostate cancer]. *Pol Merkur Lekarski* 33, 163-167.

Stewart T M, Liu D Y, Garrett C, Brown E H & Baker H W. (2009) Recruitment bias in studies of semen and other factors affecting pregnancy rates in fertile men. *Hum Reprod* 24, 2401-2408.

Storey C & Chopra I. (2001) Affinities of beta-lactams for penicillin binding proteins of *Chlamydia trachomatis* and their antichlamydial activities. *Antimicrob Agents Ch* 45, 303-305.

Su H & Caldwell H D. (1995) CD4+ T cells play a significant role in adoptive immunity to *Chlamydia trachomatis* infection of the mouse genital tract. *Infect Immun* 63, 3302-3308.

Suarez S S & Pacey A A. (2006) Sperm transport in the female reproductive tract. *Hum Reprod Update* 12, 23-37.

Sutcliffe S, Giovannucci E, Gaydos C A, Viscidi R P, Jenkins F J, Zenilman J M, Jacobson L P, De Marzo A M, Willett W C & Platz E A. (2007) Plasma antibodies against Chlamydia trachomatis, human papillomavirus, and human herpesvirus type 8 in relation to prostate cancer: a prospective study. *Cancer Epidemiol Biomarkers Prev* 16, 1573-1580.

Svenstrup H F, Fedder J, Abraham-Peskir J, Birkelund S & Christiansen G. (2003) *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 18, 2103-2109.

Svenstrup H F, Fedder J, Kristoffersen S E, Trolle B, Birkelund S & Christiansen G. (2008) *Mycoplasma genitalium*, *Chlamydia trachomatis*, and tubal factor infertility--a prospective study. *Fertil Steril* 90, 513-520.

Sweet R L, Landers D V, Walker C & Schachter J. (1987) *Chlamydia trachomatis* infection and pregnancy outcome. *Am J Obstet Gynecol* 156, 824-833.

Talebi A R, Moein M R, Tabibnejad N & Ghasemzadeh J. (2008) Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests. *Andrologia* 40, 245-251.

Taraktchoglou M, Pacey A A, Turnbull J E & Eley A. (2001) Infectivity of Chlamydia trachomatis serovar LGV but not E is dependent on host cell heparan sulfate. *Infect Immun* 69, 968-976.

Taylor-Robinson D. (1983) The role of mycoplasmas in non-gonococcal urethritis: a review. *Yale J Biol Med* 56, 537-543.

Taylor-Robinson D, Furr P M & Hanna N F. (1985) Microbiological and serological study of non-gonococcal urethritis with special reference to *Mycoplasma genitalium*. *Genitourin Med* 61, 319-324.

Taylor-Robinson D & Manchee R J. (1967) Novel approach to studying relationships between mycoplasmas and tissue culture cells. *Nature* 216, 1306-1307.

Taylor-Robinson D & Thomas B J. (1991) Detection of *Chlamydia pneumoniae*. *Lancet* 338, 321-322.

Taylor B D, Darville T, Ferrell R E, Kammerer C M, Ness R B & Haggerty C L. (2012) Variants in toll-like receptor 1 and 4 genes are associated with Chlamydia trachomatis among women with pelvic inflammatory disease. *J Infect Dis* 205, 603-609.

Taylor B D & Haggerty C L. (2011) Management of *Chlamydia trachomatis* genital tract infection: screening and treatment challenges. *Infect Drug Resist* 4, 19-29.

Taylor M L, Mainous A G & Wells B J. (2005) Prostate cancer and sexually transmitted diseases: A meta-analysis. *Fam Med* 37, 506-512.

The Practice Committee of American society for Reproductive Medicine (ASRM) (2013) The clinical utility of sperm DNA integrity testing: a guideline. *Fertil Steril*

Thomas B J, MacLeod E J & Taylor-Robinson D. (1993) Evaluation of sensitivity of 10 diagnostic assays for Chlamydia trachomatis by use of a simple laboratory procedure. *J Clin Pathol* 46, 912-914.

Thylefors B. (1995) Global trachoma control past, present and future approaches. Revue internationale du trachome et de pathologie oculaire tropicale et subtropicale et de sante publique : organe de la Ligue contre le trachome avec la collaboration de l'International Organization against Trachoma et des organisations nationales et internationales de sante publique 72, 13-80.

Tiitinen A, Surcel H M, Halttunen M, Birkelund S, Bloigu A, Christiansen G, Koskela P, Morrison S G, Morrison R P & Paavonen J. (2006) *Chlamydia trachomatis* and chlamydial heat shock protein 60-specific antibody and cell-mediated responses predict tubal factor infertility. *Hum Reprod* 21, 1533-1538.

Tilton R C, Judson F N, Barnes R C, Gruninger R P, Ryan R W & Steingrimsson O. (1988) Multicenter comparative evaluation of two rapid microscopic methods and culture for detection of *Chlamydia trachomatis* in patient specimens. *J Clin Microbiol* 26, 167-170.

Totten P A, Schwartz M A, Sjostrom K E, Kenny G E, Handsfield H H, Weiss J B & Whittington W L. (2001) Association of *Mycoplasma genitalium* with nongonococcal urethritis in heterosexual men. *J Infect Dis* 183, 269-276.

Treharne J D, Ripa K T, Mardh P A, Svensson L, Westrom L & Darougar S. (1979) Antibodies to *Chlamydia trachomatis* in acute salpingitis. *Br J Vener Dis* 55, 26-29.

Tremellen K & Tunc O. (2010) Macrophage activity in semen is significantly correlated with sperm quality in infertile men. *Int J Androl* 33, 823-831.

Tsunoe H, Tanaka M, Nakayama H, Sano M, Nakamura G, Shin T, Kanayama A, Kobayashi I, Mochida O, Kumazawa J & Naito S. (2000) High prevalence of *Chlamydia trachomatis*, Neisseria gonorrhoeae and *Mycoplasma genitalium* in female commercial sex workers in Japan. *Int J STD AIDS* 11, 790-794.

Tully J G, Taylor-Robinson D, Cole R M & Rose D L. (1981) A newly discovered mycoplasma in the human urogenital tract. *Lancet* 1, 1288-1291.

Uldum S A, Jensen J S, Sondergard-Andersen J & Lind K. (1992) Enzyme immunoassay for detection of immunoglobulin M (IgM) and IgG antibodies to *Mycoplasma pneumoniae*. *J Clin Microbiol* 30, 1198-1204.

Uno M, Deguchi T, Komeda H, Hayasaki M, Iida M, Nagatani M & Kawada Y. (1997) *Mycoplasma genitalium* in the cervices of Japanese women. *Sex Transm Dis* 24, 284-286.

Uuskula A & Kohl P K. (2002) Genital mycoplasmas, including *Mycoplasma genitalium*, as sexually transmitted agents. *Int J STD AIDS* 13, 79-85.

van den Brule A J, Hemrika D J, Walboomers J M, Raaphorst P, van Amstel N, Bleker O P & Meijer C J. (1993) Detection of Chlamydia trachomatis in semen of artificial insemination donors by the polymerase chain reaction. *Fertil Steril* 59, 1098-1104.

van Valkengoed I G M, Morre S A, van den Brule A J C, Meijer C J L M, Bouter L M & Boeke A J P. (2004) Overestimation of complication rates in evaluations of Chlamydia trachomatis screening programmes - implications for cost-effectiveness analyses. *Int J Epidemiol* 33, 416-425.

van Valkengoed I G M, Postma M J, Morre S A, van den Brule A J C, Meijer C J L M, Bouter L M & Boeke A J P. (2001) Cost effectiveness analysis of a population based screening programme for asymptomatic Chlamydia trachomatis infections in women by means of home obtained urine specimens. *Sex Transm Infect* 77, 276-282.

Van Voorhis W C, Barrett L K, Sweeney Y T, Kuo C C & Patton D L. (1996) Analysis of lymphocyte phenotype and cytokine activity in the inflammatory infiltrates of the upper genital tract of female macaques infected with *Chlamydia trachomatis*. *J Infect Dis* 174, 647-650.

Van Waart J, Kruger T F, Lombard C J & Ombelet W. (2001) Predictive value of normal sperm morphology in intrauterine insemination (IUI): a structured literature review. *Hum Reprod Update* 7, 495-500.

Vigil P, Morales P, Tapia A, Riquelme R & Salgado A N. (2002) *Chlamydia trachomatis* infection in male partners of infertile couples: incidence and sperm function. *Andrologia* 34, 155-161.

Villegas H, Pinon M, Shor V & Karchmer S. (1991) Electron microscopy of *Chlamydia trachomatis* infection of the male genital tract. *Arch Androl* 27, 117-126.

Wallace L A, Scoular A, Hart G, Reid M, Wilson P & Goldberg D J. (2008) What is the excess risk of infertility in women after genital chlamydia infection? A systematic review of the evidence. *Sex Transm Infect* 84, 171-175.

Wan C C, Wang H, Hao B J, Shang X J & Huang Y F. (2003) [Infection of *Chlamydia trachomatis* and apoptosis of spermatogenic cells]. *Zhonghua Nan Ke Xue* 9, 350-351, 354.

Wang R Y, Grandinetti T, Shih J W, Weiss S H, Haley C L, Hayes M M & Lo S C. (1997) *Mycoplasma genitalium* infection and host antibody immune response in

patients infected by HIV, patients attending STD clinics and in healthy blood donors. *FEMS Immunol Med Microbiol* 19, 237-245.

Wang S P & Grayston J T. (1970) Immunologic Relationship between Genital Tric, Lymphogranuloma Venereum, and Related Organisms in a New Microtiter Indirect Immunofluorescence Test. *Am J Ophthalmol* 70, 367-&.

Wang Y. (1999) Etiology of trachoma: a great success in isolating and cultivating *Chlamydia trachomatis*. *Chin Med J (Engl)* 112, 938-941.

Watson E J, Templeton A, Russell I, Paavonen J, Mardh P A, Stary A & Pederson B S. (2002) The accuracy and efficacy of screening tests for *Chlamydia trachomatis*: a systematic review. *J Med Microbiol* 51, 1021-1031.

Westers H, Dorenbos R, van Dijl J M, Kabel J, Flanagan T, Devine K M, Jude F, Seror S J, Beekman A C, Darmon E, Eschevins C, de Jong A, Bron S, Kuipers O P, Albertini A M, Antelmann H, Hecker M, Zamboni N, Sauer U, Bruand C, Ehrlich D S, Alonso J C, Salas M & Quax W J. (2003) Genome engineering reveals large dispensable regions in Bacillus subtilis. *Mol Biol Evol* 20, 2076-2090.

Westrom L. (1975) Effect of acute pelvic inflammatory disease on fertility. *Am J Obstet Gynecol* 121, 707-713.

Westrom L, Bengtsson L P & Mardh P A. (1981) Incidence, trends, and risks of ectopic pregnancy in a population of women. *Br Med J (Clin Res Ed)* 282, 15-18.

Westrom L, Joesoef R, Reynolds G, Hagdu A & Thompson S E. (1992) Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sex Transm Dis* 19, 185-192.

Westrom L V. (1994) Sexually transmitted diseases and infertility. *Sex Transm Dis* 21, S32-37.

Wiggins R, Horner P J, Whittington K & Holmes C H. (2009) Quantitative analysis of epithelial cells in urine from men with and without urethritis: implications for studying epithelial: pathogen interactions in vivo. *BMC Res Notes* 2, 139.

Wills G S, Horner P J, Reynolds R, Johnson A M, Muir D A, Brown D W, Winston A, Broadbent A J, Parker D & McClure M O. (2009) Pgp3 antibody enzyme-linked immunosorbent assay, a sensitive and specific assay for seroepidemiological analysis of *Chlamydia trachomatis* infection. *Clin Vaccine Immunol* 16, 835-843.

Wisniewski C A, White J A, Michel C E, Mahilum-Tapay L, Magbanua J P, Nadala E C, Jr., Barber P J, Goh B T & Lee H H. (2008) Optimal method of collection of first-void urine for diagnosis of *Chlamydia trachomatis* infection in men. *J Clin Microbiol* 46, 1466-1469.

Witkin S S, Askienazy-Elbhar M, Henry-Suchet J, Belaisch-Allart J, Tort-Grumbach J & Sarjdine K. (1998) [Circulating antibodies against an epitope of the heat-shock protein 60kDA of *chlamydia trachomatis* in infertile couples. Relation with surface chlamydia trachomatis anti-antigen antibody and E. coli anti-HSP60 antibody and human anti-HSP60]. *Contracept Fertil Sex* 26, suppl I-VI.

Witkin S S, Kligman I & Bongiovanni A M. (1995) Relationship between an asymptomatic male genital tract exposure to *Chlamydia trachomatis* and an autoimmune response to spermatozoa. *Hum Reprod* 10, 2952-2955.

World Health Organisation (1999) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, Cambridge.

World Health Organisation (2010) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, Cambridge.

Wolner-Hanssen P & Mardh P A. (1984) In vitro tests of the adherence of *Chlamydia trachomatis* to human spermatozoa. *Fertil Steril* 42, 102-107.

Wong P C, Balmaceda J P, Blanco J D, Gibbs R S & Asch R H. (1986) Sperm washing and swim-up technique using antibiotics removes microbes from human semen. *Fertil Steril* 45, 97-100.

Wyrick P B, Knight S T, Paul T R, Rank R G & Barbier C S. (1999) Persistent chlamydial envelope antigens in antibiotic-exposed infected cells trigger neutrophil chemotaxis. *J Infect Dis* 179, 954-966.

Wyrobek A J, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs E W, Glaser R L, Pearson F S & Evenson D. (2006) Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proc Natl Acad Sci U S A* 103, 9601-9606.

Yeganeh O, Jeddi-Tehrani M, Yaghmaie F. (2013) A survey on the prevalence of *Chlamydia trachomatis* and *Mycoplasma genitalium* infections in symptomatic and asymptomatic men, Tehran, Iran. *International Red Crescent Medical Journal* 15, 340-344 .

Yoshida H, Kishi Y, Shiga S & Hagiwara T. (1998) Differentiation of Chlamydia species by combined use of polymerase chain reaction and restriction endonuclease analysis. *Microbiol Immunol* 42, 411-414.

Yoshida T, Deguchi T, Ito M, Maeda S, Tamaki M & Ishiko H. (2002) Quantitative detection of *Mycoplasma genitalium* from first-pass urine of men with urethritis and asymptomatic men by real-time PCR. *J Clin Microbiol* 40, 1451-1455.

Zini A, Bielecki R, Phang D & Zenzes M T. (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 75, 674-677.

9 Appendix I

<table>
<tr><td rowspan="5">Research and Clinical Centre For Infertility
Shahid Sadughi University, YazD

The role of C. trachomatis and M. genitalium in Infertile couples.</td><td>Study number</td><td></td><td>Author</td><td>Laleh Marvast</td></tr>
<tr><td>Version</td><td>1.1</td><td rowspan="4">Page</td><td rowspan="4">1 of 4</td></tr>
<tr><td>Date</td><td></td></tr>
<tr><td>Authorized By:</td><td></td></tr>
<tr><td>Review date:</td><td></td></tr>
<tr><td colspan="5">Title: Data Collection Form</td></tr>
</table>

PARTICIPANT DETAILS

Couple number	
DOB♂	
DOB♀	
Hospital No♂	
Hospital No♀	
Referring Trust:	Abarkuh ◯ Ardakan ◯ Bafgh ◯ Meibod ◯ Mehreez ◯ Taft ◯ Yazd ◯ Other ◯

Date form completed:	
Couple number:	

Obstetric History

Infertility	Primary ◯ Secondary ◯
Date of last pregnancy	
History of Abortion	Yes ◯ No ◯
Etiology	Cervical insufficiency ◯ Immunological ◯ Genetic ◯ Infection ◯ Anatomic ◯ Other ◯
Date:	
History of ART Assisted Reproductive Technology	Yes ◯ No ◯
Contraception:	Hormonal ◯ IUD ◯ Natural ◯ Condom ◯
Duration:	

Menstural Pattern:	Regular ◯ Irregular ◯
Etiology:	Anovulatory Cycle ◯ Endometriosis ◯ Endocrine problem: Thyroid ◯ PCOS ◯ Obesity ◯ Anorexia ◯
Diagnostic test:	Ultrasonography ◯ Biopsy ◯ Laparoscopy ◯ Hysteroscopy◯
Result:	
Treatment:	Progesterone ◯ Estrogene ◯ Combined ◯ Induction of Ovulation◯ Other ◯

History of Infection: (PID)♀	Yes ◯ No ◯
Treatment	Yes ◯ No ◯
Date:	
History of Infection:♂	Urethritis ◯ Orchitis◯ Prostatitis ◯
Treatment	Yes ◯ No ◯
Date:	

General History (♂)

History of Surgery :(♂)	Abdominal ◯ Pelvic ◯ Reversal Vasectomy ◯ Bladder◯Cryptorchidism ◯
Date:	
History of Trauma	Yes ◯ No ◯
Drugs	Alcohol ◯ Nicotin◯ Cannabis ◯ Opium ◯ Other ◯
Infectious disease	TB ◯ Febrile condition ◯ STD ◯ Other ◯
Date	
Malignancy	Yes ◯ No ◯
Site	GUT ◯ Non- GUT ◯
Treatment	Chemotherapy◯ Radiotherapy ◯ Combined◯
Site of Radiotherapy:	
Date	
Occupation	
Endocrine Problem	

General History (♀)

History of Surgery :(♀)	Abdominal ◯ Pelvic ◯
Date:	
History of Trauma	Yes ◯ No ◯
Drugs	Alcohol ◯ Nicotin◯ Cannabis ◯ Opium ◯ Other ◯
Infectious disease	TB ◯ STD ◯ Other ◯
Date	
Malignancy	Yes ◯ No ◯
Site	GUT ◯ Non- GUT ◯
Treatment	Chemotherapy◯ Radiotherapy ◯ Combined◯
Site of Radiotherapy:	
Date	
Occupation	
Endocrine Problem	Thyroid ◯ Galactorrhoea ◯ Hyperprolactinemia ◯ PCOS ◯

Follow up (Infertile Couples)

Diagnostic test:	Ultrasonography ◯ Biopsy ◯ Laparoscopy ◯ Hysteroscopy◯ HSG ◯
Result:	
Treatment(after diagnose)	Antibiotic ◯ Hormonal ◯ Surgery ◯ ART* ◯
ART* Assisted Reproductive Technology	IVF ◯ ICSI ◯ IUI ◯ Zift ◯
Pregnancy	Normal ◯ ART ◯
Embryo quality	
Success Rate	
Miscarriage	Yes ◯ No ◯
Delivery	Vaginal ◯ C/S ◯

10 Appendix II

The University Of Sheffield.

The Medical School.

Ms Jean Lazenby
Research Ethics Administrator
Room EU28, Medical School
Beech Hill Road
Sheffield
S10 2RX
Telephone: +44 (0) 114 271 2237
Fax: +44 (0) 114 271 3892
Email: j.lazenby@sheffield.ac.uk

12 October 2010

Dr A R Eley
Department of Infection & Immunity
Medical School
Beech Hill Road
SHEFFIELD S10 2RX

Dear Adrian

PROJECT TITLE: 'The role of Chlamydia trachomatis & Mycoplasma genitalium in infertile couples from Iran' – SMBRER147

I am pleased to inform you that on 11 October 2010 the School's Ethics Reviewers **approved** the above-named project on ethics grounds, on the basis that you will adhere to and use the following documents that you submitted for ethics review.

i) Ethics form [approved – 11 October 2010]
ii) Patient information sheet-controls [approved – 11 October 2010]
iii) Patient information sheet-patients [approved – 11 October 2010]
iv) Protocol [approved – 11 October 2010]
v) Data collection form (Yazd) [approved – 11 October 2010]
vi) Data collection form for fertile women [approved – 11 October 2010]
vii) Enrolment log [approved – 11 October 2010]
viii) Screening log [approved – 11 October 2010]

If during the course of the project you need to deviate from the above-approved documents please inform me. The written approval of the School's Ethics Review Panel will be required for significant deviations form or significant changes to the above-approved documents. If you decide to terminate the project prematurely please inform me.

Yours sincerely

Jean Lazenby
School Research Ethics Administrator

The Queen's Anniversary Prizes
For Higher and Further Education
1998 2000 2002

Yazd Research &Clinical Centre for Infertility

No=1084

Date: 27/8/2009

The meeting was held in IVF centre, that was 68th research committee, on Wednesday (26/8/2009).Those are present in this meeting: Prof Aflatoonian, Dr Ghasemi, Dr Davar, Dr Abdoli, Dr Mohamad hasan Sheikhha, Dr Dehghani, Dr Anvary.

There are presented some research proposal by investigators or their supervisor. One of research proposal that was presented by Prof Aflatoonian, which is a joint program between The University of Sheffield and Shahid Sadughi Medical University of Yazd.

The title of research proposal: Testing for *Chlamydia trachomatis* and/or *Mycoplasma genitalium* including serology to determine pregnancy rates in infertile couples from Yazd. The investigator is Dr Laleh Dehghan Marvast who is a PhD student in The University of Sheffield, and this research is a part of her PhD.

This project was approved by committee, also because of sending samples to UK, it needs to be approved by Ministry of Health Ethic Committee.

This is a translation of pages which follow.

No=686/P88S

Date: 2/1/2010

To: Dr Fatemeh Ezaaddini, Research Deputy, Yazd Medical University, Yazd

From: Dr Kazem Zendedel, Secretary of Ethic Committee for Medical Research, Tehran

Ethic Committee for Medical research

Ethic Committee approval

Declaration no=11P/88k-A Date: 9/9/2009

Inspected at: Ministry of Health Ethic Committee Date: 23/12/2009

Decision: conditional acceptance

Inspection type: regular

This project has been approved in 46th meeting of Ethic Committee for Medical research if only all of experiments carry out on samples that have been mentioned in proposal and the remainder of samples must be discarded.

Title of research project: Testing for *Chlamydia trachomatis* and/or *Mycoplasma genitalium* including serology to determine pregnancy rates in infertile couples from Yazd.

Investigator details:

Name: Dr Laleh Dehghan Marvast

E-mail: Laleh.dm236@gmail.com

Tel: 0351-8247085

Address: Yazd Research &Clinical Centre for Infertility

This is a translation of pages which follow.

11 Appendix III

Sample size calculation:

Type 1 error (alpha): 5%.

Power: 80% (probability you reject the null when it is false).

There are 3 assumptions:

Infection rate in fertile couples:	4%	8%	11%
Infection rate in infertile couples:	8%	12%	15%
Number of couples (patients) required:	65 (130)	199(398)	253(506)

These sets of infection rates give the population prevalence of 12% if 15% of couples are infertile.

For a quantitative measure (comparing two groups) standardised mean difference of 0.5 standard deviations would require 64 in each group. Standardised mean of difference of .28 would require 202 in each group.

Statistical advice was provided by Dr. Dawn Teare who used the reference blew: Sample Size Tables for clinical studies. Third edition, Machin D, Campbell MJ, Tan SB, Tan SH. Wiley-Blackwell 2009.

12 Appendix IV

Prevalence of *C. trachomatis* in different studies.

References	**Specimen**	**Method**	**Population**	**Country**	**Prevalence**
Domes *et al*., 2012	Urine & semen	SDA	Men (infertile)	Canada	0.3%
de Lima Freitas *et al.,* 2011	Endocervical	PCR	Women (infertile)	Brazil	52.8%
Mahmud *et al*., 2011	Serum	Serology	Women (young)	Bangladesh	4.6%
Mahmud *et al*., 2011	Serum	Serology	Women (pregnant)	Bangladesh	24.1%
Ikeme *et al*., 2011	Serum	Serology	Women (different age)	Nigeria	29.4%
Idahl *et al*., 2004	Urine & semen	PCR	Infertile men	Sweden	7.1%
Idahl *et al*., 2004	Urine	PCR	Infertile women	Sweden	6.8%
Idahl *et al*., 2004	Serum	Serology	Infertile men	Sweden	20.1%
Idahl *et al*., 2004	Serum	Serology	Infertile women	Sweden	24.2%
Eggert-Kruse *et al*., 2003	Urine	PCR	Men (infertile)	Germany	1.8%
Eggert-Kruse *et al*., 2003	Urine	PCR	Women (subfertile)	Germany	1.0%
Bollmann *et al*., 2001	Semen	LCR	Men (infertile)	Germany	4.0%
Eggert-Kruse *et al*., 1997	Semen	LCR	Men (infertile)	Germany	0.0%
Eggert-Kruse *et al*., 1997	Serum	Serology	Men (subfertile)	Germany	12.6%
Eggert-Kruse *et al*., 1997	Serum	Serology	Women (subfertile)	Germany	20.8%